NATURAL KILLER-LIKE CELLS IN PERIPHERAL BLOOD AND UTERINE ENDOMETRIUM IN SHEEP: CHARACTERISTICS AND REGULATION BY PREGNANCY-ASSOCIATED PROTEINS

By

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I dedicate this thesis to my dear father, mother, wife, son and daughter.

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LIST OF ABBREVIATIONS

ADCC	Antibody-Dependent Cell-Mediated Cytotoxicity
BHV-1	Bovine Herpes Virus-1
BSA	Bovine Serum Albumin
ConA	Concanovalin A
CRA	Chromium Release Assay
CSF	Colony Stimulating Factor
DPBS	Dulbecco's Phosphate Buffered Saline
EAE	Experimental Allergic Encephalomyelitis
EEC	Endometrial Epithelial Cells
EDTA	Ethylenediamine Tetraacetic Acid
FAM	Function Associated Molecule
GMC	Granulated Metrial Gland Cell
GM-CSF	Granulocyte-Macrophage-Colony Stimulating Factor
HLA	Human Leukocyte Antigen
HMWGP	High Molecular Weight Glycoprotein
IEL	Intraepithelial Lymphocytes
IFN	Interferon
IL	Interleukin
IL-2R	Interleukin-2 Receptor
INOS	Inducible Nitric Oxide Synthase

ITIM	Immunoreceptor Tyrosine-Based Inhibition Motif
KIR	Killing Inhibitory Receptors
LAK	Lymphokine Activated Killer
LGL	Large Granular Lymphocytes
LIF	Leukaemia Inhibitory Factor
MEM	Minimal Essential Medium
MHC	Major Histocompatibility Complex
NK	Natural Killer Cell
NKIM	NK Inhibitory Molecules
NO	Nitric Oxide
OVAL	Ovalbumin
OvUS	Ovine Uterine Serpin
PBL	Peripheral Blood Lymphocytes
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PHA	Phytohemagglutinin
PIBF	Progesterone Induced Blocking Factor
PolyI•PolyC	Polyinosinic-Polycytidylic Acid
RAG	Recombination Activating Genes
RSA	Recurrent Spontaneous Abortion
SCID	Severe Combined Immunodeficiency
SERPIN	Serine Proteinase Inhibitor
STOT2	Saban Tekin Ovine Trophoblast Cells-2

TCID	Tissue Culture Infectious Dose
TCR	T Cell Receptor
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor

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NATURAL KILLER-LIKE CELLS IN PERIPHERAL BLOOD AND UTERINE ENDOMETRIUM IN SHEEP: CHARACTERISTICS AND REGULATION BY PREGNANCY-ASSOCIATED PROTEINS

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Natural Killer (NK) cells have been found in uterine tissues of humans, mice and pigs. Moreover, these cells can lyse trophoblast cells following interleukin-2 (IL-2) stimulation. In sheep, ovine uterine serpin (OvUS) secreted by the endometrium and interferon-τ (IFN-τ) secreted from the preattachment trophoblast may contribute to the protection of the allogeneic conceptus by regulating lytic function of NK cells during pregnancy. The objectives of this dissertation were to study presence, cytotoxic function, mechanism of cytotoxicity, and regulation of NK-like cells during pregnancy in the sheep. Cells in peripheral blood (PBL) and endometrial epithelium (EEC) lysed a putative NK target, the D17 cell. Lysis was increased by infection of target cells with bovine herpes virus-1 (BHV-1) and by incubation of effector cells with IL-2. In general, sheep primary trophoblast cells were resistant to lysis by NK-like cells from third party even in the presence of IL-2 and BHV-1. In some cases, maternal PBL displayed cytotoxic activity against their own trophoblast. These data suggest that in most

pregnancies trophoblast is resistant to lysis by NK-like cells but that specific maternal cytotoxic lymphocytes can be generated in some pregnancies. In contrast to primary cultures of trophoblast, PBL exerted NK-like lysis against subcultured trophoblast cells, suggesting that de-differentiation of the trophoblast made them susceptible to NK-like lysis. The lysis of PBL and EEC was also immunoneutralized by anti-function associated molecule (FAM), an antibody recognizing NK cells, indicating that NK-like cells in PBL and EEC are anti-FAM+. In addition, NK-like lytic activity of PBL was blocked by molecules such as concanomycin-A, NH_4Cl, leupeptin, and pepstatin-A that inhibit acidification of lytic granules and block enzymatic processing of perforin. This result suggests that the cytotoxic activity of NK-like cells in PBL is mediated by perforin-like lytic molecules. The NK-like lytic activity of both PBL and EEC was inhibited by OvUS; thus, OvUS may be involved in immunosuppression of NK-like cell responses during pregnancy. In contrast, ovine and bovine IFN-τ (OvIFN-τ, BoIFN-τ) increased NK-like lytic activity of PBL, suggesting IFN-τ can activate NK-like cells during early pregnancy. Overall, these results imply that while down-regulation of major histocompatibility (MHC) class I molecules makes the sheep conceptus at risk for attack by NK-like cells, several mechanisms exist to achieve the survival of the conceptus in the face of this risk. These mechanisms include 1) differentiation of the trophoblast to make it resistant to killing and 2) suppression of NK cells by OvUS. The fact that IFN-τ enhances lytic activity of NK cells may mean that either activation of NK cells early in pregnancy is beneficial for conceptus survival or the other molecules prevent this activation in utero.

CHAPTER 1
PREFACE

During pregnancy, maternal immune responses are regulated to prevent maternal rejection of the allogenic conceptus. Immunologic regulation is mediated by hormones, immunosuppressive cells and their products, and secreted products of non-lymphoid tissues of the uterus and conceptus. Improper immune regulation or interactions between conceptus and maternal cells at the maternal-fetal interface can lead to abortion.

One cell type that may be an important determinant of the survival of the conceptus is the Natural Killer (NK) cell. Cells of this phenotype have been found in uterine tissues of humans, mice and pigs (Parr et al. 1990, Croy et al. 1991, King et al. 1991, Clark et al. 1994, Yu et al. 1994, King et al 1996). Although mouse and human placental cells are generally resistant to NK cell lysis (Zuckermann et al. 1987, King and Loke 1990), interleukin (IL)-2–activated NK cells, lymphokine activated killer (LAK) cells, can lyse trophoblast (King and Loke 1990). Spontaneous abortions seen in the DBA/2xCBA/j abortion prone mouse model are associated with infiltration of NK cells into implantation sites (Drake et al. 1989, Kinsky et al. 1990) and the neutralization of NK cells by anti-asialoGM1 prevented abortion (Chaouat et al. 1990). There is also increased infiltration of NK cells at the decidua of women experiencing spontaneous abortion (Kwak et al. 1998).

The role of NK cells during pregnancy in sheep is problematic. Indeed, the existence of NK cells in ruminants has been questionable. Unlike human and mouse NK cells, sheep peripheral blood lymphocytes (PBL) cannot lyse traditional NK cell targets in a 4 h

^{51}Chromium (^{51}Cr) -release assay (CRA). Even in a 20 h CRA, PBL exert very low levels of lysis against K562 cells (Segerson and Gunsett 1994). The only NK-reactive antibody that has been reported to react with sheep and cattle is a monoclonal antibody developed against function-associated molecule (anti-FAM or NK5C6), which has been shown to be expressed in mice and human NK cells (Evans et al. 1988, Harris et al. 1993). It has been shown that 12% of sheep lymphocytes reacted with this antibody (Evans et al. 1988, Harris et al. 1993). Despite these negative findings, others studies suggest the existence of NK-like cells in sheep (Evans et al. 1988, Harris et al. 1993, Segerson and Gunsett 1994). For example, IL-2–treated PBL could lyse cells recovered from Day 16 and 19 conceptus tissues (Segerson and Gunsett 1994). In addition, the expression of perforin mRNA in sheep endometrial intraepithelial lymphocytes (IEL) has been reported (Fox and Meeusen 1999).

In sheep, pregnancy is associated with changes in the numbers of endometrial IEL. Numbers of γδ T cells in luminal epithelium of the interplacentomal endometrium increase during mid and late pregnancy, however, numbers of the other lymphocytes decrease in glandular and luminal epithelium (Lee et al. 1992). In addition, Liu et al. (1997) reported that γδ T IEL become activated during late pregnancy and this activation depends on local presence of the conceptus. In contrast to an earlier study, mRNA for CD25 is never expressed on γδ T cells and in the endometrium during pregnancy (Fox et al. 1998, Majewski et al. 2001). In fact, the systemic effect of pregnancy rather than local stimulation of conceptus caused the increase in numbers of γδ T cells during late pregnancy (Majewski et al. 2001).

Several regulatory molecules produced during pregnancy have been implicated in the regulation of immune function in sheep. Progesterone, which is produced initially only by the corpus luteum and later by the placenta (Casida and Warwick 1945, Beal et al. 1986), may be an important hormone for allograft survival during pregnancy. Progesterone can directly inhibit lymphocyte function at high concentrations (Low and Hansen 1988) and progesterone treatment reduced CD45R+ and major histocompatibility (MHC) class-II+ cells in the uterus (Gottshall and Hansen 1992). Moreover, progesterone prevented rejection of intrauterine skin grafts (Hansen et al. 1986) and intrauterine grafting of mouse hybridoma cells (Majewski and Hansen 1999). It has been suggested that progesterone regulates uterine immune function indirectly by inducing secretion of immunosuppressive proteins from the uterine endometrium (Hansen and Liu 1996). The best studied of those, ovine uterine serpin (OvUS), is a member of the serine proteinase inhibitor family that can inhibit mixed lymphocyte reaction, mitogen and antigen-induced proliferation of lymphocytes (Segerson et al. 1984, Stephenson et al. 1989, Skopets and Hansen, 1993, Skopets et al. 1995). Ovine uterine serpin can also inhibit NK cell activity in sheep and mice (Liu and Hansen 1993, Skopets and Hansen 1995). Placental secretory molecules may also involve uterine immunoregulation. The best studied of these is interferon (IFN) -τ, which is secreted by trophoblast between Day 12 through Day 21 of pregnancy in sheep and cattle (Hansen et al. 1988, Farin et al. 1990, Martal et al. 1998), and which has many biological activities including inhibition of mitogen-induced proliferation of lymphocytes (Newton et al. 1989, Skopets et al. 1992).

There is some conflicting evidence regarding whether pregnancy can alter the immune system systemically. Blood from pregnant cows during very early pregnancy was

inhibitory to adoptive transfer of contact sensitivity in mice (Klima 1985). In mid-pregnancy, blood from cows was less able to support lymphocyte proliferation in culture than blood from nonpregnant cows or cows earlier or later in pregnancy (Manak 1982, Winter et al. 1986). In contrast, lymphocytes from pregnant cows were not suppressed in lymphocyte proliferation tests as compared with lymphocytes from nonpregnant cows (Manak 1982, Winter et al. 1986, Monterroso and Hansen 1993). Also pregnancy did not delay skin graft rejection in cattle (Billingham and Lampkin 1957). It is recently reported that allospecific cytotoxic T lymphocyte (CTL) responses are inhibited during pregnancy in mice (Tafuri et al. 1995). In human, progesterone stimulates secretion of progesterone-induced blocking factor (PIBF) from PBL, which inhibits NK cell function and 60% of decidual NK cell express PIBF during pregnancy (Szekeres-Bartho et al. 2001).

The major goal of this dissertation is to understand regulation of NK-like cytotoxic lymphocytes during ovine pregnancy. Therefore, experiments were designed to determine 1) whether sheep trophoblast is susceptible to lysis by maternal immune cells in peripheral blood, 2) whether peripheral blood lymphocytes and lymphocytes in endometrium exhibit cytotoxicity against NK cell targets, 3) whether these cytotoxic cells have properties characteristic of NK cells, 4) whether natural killer-like cells are regulated by pregnancy associated molecules, OvUS and IFN-τ.

CHAPTER 2
REVIEW OF LITERATURE

Introduction

Upon recognition of a tissue graft, the immune system responds by mounting either a tolerizing or rejection reaction. Generally, an allogeneic tissue graft transplanted into an immunologically-normal recipient is rejected by the host immune system. As an allogeneic graft that expresses transplantation antigens inherited from its father (Gill 1985), the mammalian conceptus is also at risk from maternal anti-conceptus immune responses elicited through recognition of alloantigens by maternal effector cells. That the allogeneic conceptus usually survives in the uterus despite this threat has been termed the immunological paradox of pregnancy (Medawar 1953). In a seminal paper, Medawar (1953) postulated several hypotheses to explain how the conceptus escapes maternal immune attack. These hypotheses include (i) the trophoblast serving as an anatomic barrier between mother and fetus, (ii) the uterus being an immunologically-privileged site, (iii) antigenic immaturity of the conceptus, (iv) immunologic tolerance by the maternal immune system, and (v) immunosuppression at the maternal-fetal interface.

Since this paper, extensive research has been carried out to evaluate the relevance of these hypotheses. Although the trophoblast separates the uterus from the fetus, it is not a complete barrier to immune cells because maternal lymphocytes can pass into the fetus and vice versa (Chaouat et al. 1983, Zhang and Miller 1993, Pitrowski and Croy 1996). Offspring of severe combined immunodeficiency (SCID) mice that had been injected with PBL mounted immune response against dinitrophenyly-keyhole limpet

hemocyanin, suggesting that grafted leukocytes had crossed the placenta (Greenwood et al. 1996).

Immunologically-privileged sites such as brain and testis are the host tissues that allow graft survival without immunosuppression (reviewed by Rossini et al. 1999). In contrast, skin grafts placed in the uterus of the rat (Head and Billingham 1985) and sheep (Hansen et al. 1986), are rejected, suggesting that the components of immune system required for tissue rejection are present in the uterus, and that in contrast to Medawar's idea, the uterus is not an immunologically privileged. In fact, there are large numbers of lymphocytes in the uterine endometrium of mice (Croy et al. 1991, Stewart 1994), humans (Bulmer et al. 1991, King et al 1996, King et al. 1998), pigs (Yu et al. 1993, Yu et al. 1994), cattle (Leung et al 1999), and sheep (Lee et al. 1992, Liu et al. 1993, Majewski et al. 2001).

Since the expression of the classical MHC class I molecules is down-regulated on trophoblast (Rossini et al. 1999), the trophoblast is less antigenic than many tissues, resistant against T cell cytotoxicity. Furthermore, trophoblast transplanted into kidney capsule of a mouse immunized against paternal MHC is not rejected (Simmons and Russel 1962). This is not the case for the fetus, because fetal tissues (Woodruff 1957) and ectoplacental core (Simmons and Russel 1962) transplanted into external sites in a pregnant rat failed to survive. While the placenta is less antigenic than many tissues, it still expresses some tissue transplantation antigens. One example is H-2, found on mouse embryos (Edidin 1964, Billington et al. 1977).

Recent studies support Medawar's "maternal tolerance" hypothesis. Maternal T cells tolerate paternal classical MHC class I alloantigens during pregnancy (Tafuri et al. 1995).

Additionally, Tafuri et al. (1995) showed that tumor cells that express H-2K^b survived during pregnancy when transplanted into H-2K^b transgenic female mice (H-2K^k) bred to males of H-2K^b genotype. In contrast, tumors were rejected during pregnancy established by other matings. In these mice, anti-H-2K^b responses were resumed after parturition. Studies with transgenic mice expressing T cell receptor (TCR) specific for H-Y antigen showed T cells specific for fetal H-Y antigens decrease during pregnancy (Jiang and Vacchio 1998). Therefore it can be proposed that lack of maternal immune tolerance and other immunoregulatory mechanisms may cause pregnancy loss.

Maternal anti-conceptus responses are also suppressed by immunosuppressive cells or molecules such as cytokines, hormones, and other bioactive molecules that are produced at the maternal-fetal interface. Indeed, suppressor lymphocytes have been found in several species including sheep and cattle (Kamel and Wood 1990, Ark et al. 1997, Segerson et al. 1998, Segerson and Beetham 1999). In mouse, deletion of transforming growth factor-β (TGF-β) producing γδ T lymphocytes by antisera increased abortion, suggesting that TGF-β+ lymphocytes suppress lymphocytes that react with trophoblast (Arck et al. 1997). In addition, cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) prevented abortion in DBA/2xCBA/J mice (Clark et al. 1994). The immunoregulatory activity of progesterone on leukocyte function has been shown in mice, humans, and sheep (Staples et al. 1983, Low and Hansen 1988, Robertson et al. 1996, Segerson et al. 1997, Griffith et al. 1997). Similarly, prostaglandin E_2 (PGE_2) suppresses lymphocyte function (Low and Hansen 1988, Parhar et al. 1989).

As mentioned previously, one of the strategies the conceptus uses to prevent immunological rejection, decreased MHC expression, also exposes to lysis by NK cells

because, NK cells ordinarily lyse cells with aberrant MHC antigen expression (Karre et al. 1986, Karre 1995). Immunological reaction against an allogeneic graft requires interaction between the innate and adaptive immune systems (Rossini et al. 1999). However, the function of NK cells in allograft rejection is unclear (Rossini et al. 1999). It is shown that athymic rats that lack T and B cells rejected hamster heart xenografts and that neutralization of NK cells by anti-asialoGM1 antiserum extended graft survival (Lin et al. 1997). This suggests that NK cells may be involved in graft rejection. Natural killer cells alone cannot reject allografts but are often present in populations of leukocytes that infiltrate a graft (Rossini et al. 1999). NK cells have been found in uterine tissues of humans (Kiso et al. 1992, Ho et al. 1996), mice (Parr et al. 1991, Croy et al. 1997) and pigs (Yu et al. 1994). The spontaneous abortions seen in the CBA/j female mouse mated to DBA/2 males (also called the CBAxDBA model or the abortion-prone mouse model) are associated with infiltration of NK cells into implantation sites (Gendron et al. 1990, Duclos et al.1994). Moreover, activation of NK cells with polyinosinic-polycytidylic acid (polyI•polyC) leads to increased abortion in mice (Kinsky et al. 1990, Liu and Hansen 1993).

Since Medawar, the immunological paradox of pregnancy has attracted interest in understanding mechanisms by which the allogeneic conceptus survives in the uterus, in the form of a potentially hostile maternal immune system. Understanding the regulation of NK cell function during pregnancy might be critical for solving pregnancy-associated problems such as recurrent abortion in humans (Chaudhury and Knapp, 2000) and retarded in utero growth and neonatal mortality in farm animals. In this chapter, general characteristics, function and regulation of NK cells during pregnancy will be reviewed.

Biology of Natural Killer Cells

Natural killer cells are an important component of innate immunity and in tumor surveillance. They have a unique ability to lyse target cells without prior exposure to antigen (Trinchieri 1989, Lanier and Philips 1992). Like T cells, NK cells also secrete various cytokines and chemokines (reviewed in Biron et al. 1999). Natural killer cells can be found in lymphoid tissues such as blood, spleen, bone marrow, and lymph nodes (Trinchieri 1989) and in non-lymphoid tissues such as uterus (Kiso et al. 1992, Guimond et al. 1996, Ho et al. 1996, Whitelaw and Croy 1996) and liver (Ljungreen and Karre 1990).

Development

Natural killer cells originate from the same lymphoid progenitor as T and B lymphocytes (Spit et al 1995). They migrate from bone marrow to the spleen and are recruited into different tissues upon activation (Trinchieri 1989). Since NK cells lack recombination activating genes (RAG) genes and do not express the TCR - CD3 complex, they are classified as non-T cells (Mombaerts et al. 1992) and develop in SCID and recombination activating genes (RAG) - mice (Hackett et al. 1986, Mombaerts et al. 1992, Shinkai et al. 1992). Natural killer cells have large granules containing lytic molecules such as perforin and granzyme for lysing target cells (Trinchieri 1989).

In general, mature human peripheral blood NK cells are CD3- CD56+ CD16- whereas mouse NK cells are CD3- NK1.1+ asialoGM1+ (Trinchieri 1989). However, there are several subsets of NK cells, each with somewhat different origins and properties (Mowbray and Croy 1996). In mice, there is a cell type called NK T cells that expresses both NK cell markers such as NK1.1 as well as the TCR-CD3 complex (Vicari and Zlotnic 1996, Bendelac et al. 1997). These cells are found in liver in very high numbers

(MacDonald 1995). Unlike classical NK cells, development of NK T cells depends on CD1, a MHC class I-like molecule that recognizes lipids (Bendelac et al. 1994, Bendelac 1995, McDonald 1995, Bendelac et al. 1997). The NK T cells are reduced in mice deficient for β2 microglobulin and CD1 (Chen et al. 1997). Like classical NK cells, NK T cells can lyse certain NK cell targets, proliferate following IL-2 stimulation and produce IFN-γ by stimulation through NK1.1 and IL-12 (reviewed by Biron 1999).

Recognition

According to the "missing-self" hypothesis, NK cells discriminate between cells with and without self MHC class I expression (Ljungreen and Karre 1990, Karre 1995). Loss of MHC class I expression, which may occur during infection or tumor formation, targets the cell for destruction by NK cells (Ljungreen and Karre 1990, Karre 1995). Unlike T cells, NK cells require neither presence of MHC class I on target cells nor prior antigen stimulation for function (Karre 1995). Indeed, MHC class I molecules expressed on target cells inhibit NK cell function (Karre 1995, Rouas-Freis et al. 1997, Dorling et al. 2000, Routes et al. 2001). For example, NK cells specifically lysed tumors without H-2 while reconstitution of H-2 provided protection (Ljunggreen and Karre 1985, Piontek et al. 1985). Furthermore, human NK cells lysed MHC class I-deficient Epstein-Barr virus - infected lymphoblastoid cell lines, but could not lyse these same target cells after transfection with certain classical human leukocyte antigen (HLA) class I genes (Shimuzu and DeMars 1989, Storkus et al. 1989).

Inhibitory NK Cell Receptors. Earlier studies indicate that NK cell recognition of polymorphic MHC class I ligands mediated by MHC class I specific membrane receptors are regulated by activatory and inhibitory signals transmitted by NK cell receptors-ligand

interaction (reviewed by Lanier 1998). Membrane receptors on NK cells that recognize MHC class I molecules on target cells have been identified and cloned (Karlhofer et al. 1992, Wagtmann et al. 1995). The NK cell MHC class I specific receptors which belong to the inhibitory receptor superfamily are C-type lectin superfamily receptors such as Ly49 (mouse and rat) and CD94/NKG2 (humans) and immunoglobulin superfamily receptors such as killer cell immunoglobulin-like receptors (KIR) (reviewed by Long 1999).

The Ly49 family of NK cell receptors recognizes polymorphic MHC class I molecules and inhibit NK cell function (Karlhofer et al. 1992). There are at least nine members of this family located within the NK complex in mouse chromosome 6 (Brown et al. 1997). The Ly49 receptors are expressed as disulfide-linked homodimers, bind $\alpha 1$ or $\alpha 2$ domains of the MHC class I molecule and require interaction of $\beta 2$-microglobulin (Daniels et al. 1994, Orihuela et al. 1996).

The CD94/NKG2 receptors are found in human NK cells and $\gamma\delta$ T cells (Aramburu et al. 1990, Aramburu et al. 1991). They are present as disulfide-linked heterodimers and bind the nonclassical HLA-E (Lazetic et al., 1996, Long 1999) and an HLA-E molecule that is loaded with HLA-G leader peptide (Vales-Gomes et al. 1999). However, studies with antiserum against CD94 and CD94/NKG2 indicate that this receptor may involve broad recognition of MHC class I molecules including HLA-A, -B, -C and -G (reviewed by Lanier 1998 and Long 1999). The NKG2 receptor is encoded by four genes (Chang et al. 1995), whereas CD94 is encoded by a single gene located on human Chromosome 12 (reviewed by Long 1999).

Killer cell immunoglobulin-like receptors bind classical HLA class I molecules such as HLA-C and B and inhibit NK cytotoxicity (reviewed by Long 1999). It is also reported that some KIR can recognize HLA-G (Gong et al. 1994). These receptors have low affinity to HLA-G molecules (reviewed by Long 1999). The KIR are encoded by at least 12 genes located on human Chromosome 19 (Dupont et al. 1997). In mice gp49A and B relate to human KIR and cloned from mast cells (reviewed by Lanier 1997). Like human KIR, mouse gp49A and B possess immunoglobulin-like domains (reviewed by Lanier 1997).

It is proposed that each human or mouse NK cell expresses two or more receptors, some of which are specific for self MHC haplotype and others specific for non-self MHC haplotypes (Lanier 1998). Except the CD94 (Chang et al. 1995), all of these inhibitory receptors have two domains- an extracellular domain for recognition and a cytoplasmic domain for signal transduction (Plougastel and Trowsdale 1996). The cytoplasmic domain contains immunoreceptor tyrosine-based inhibition motifs (ITIM) (Lanier 1997). Although the MHC class I specific inhibitory NK cell receptors differ in structure, they all inhibit NK function using a similar mechanism (reviewed by Lanier 1998). Upon association of the extracellular domain of an inhibitory receptor with a target ligand, the tyrosine residues on ITIMs are phosphorylated. The phosphorylated ITIMs in turn recruit SHP-1 and 2, which mediate inhibition. Aberrant expression of MHC class I molecules on a target cell leads to generation of positive signaling by activatory NK cell receptors that stimulate NK cell to lyse the target (reviewed by Lanier 1998). Recent studies indicate that some subtypes of MHC class I specific NK cell receptors which do not possess ITIM may participate in activation of NK cells (Lanier 1998).

Activatory NK Cell Receptors. As discussed above, NK cells also have several membrane receptors and coreceptors that activate NK cell proliferation, migration, cytotoxicity and cytokine production. The most well-described receptors are CD16 (FcγRIII), CD2, NKR-P1, and CD28 (Trinchieri 1989). Additionally, other membrane receptors such as CD69, mouse 2B4, human DNAM-1 and CD44 are involved activation of cytotoxicity (reviewed in Lanier 1998). Like inhibitory membrane receptors, activatory receptors have extracellular and cytoplasmic domains that usually contain immunoglobulin-like tyrosine-based activation motifs (ITAM) or similar motifs (reviewed by Long 1999). The association of activatory receptors with ligands expressed on target cells results in phosphorylation of tyrosine residues on ITAMs. Natural killer cell functions such as cytokine release and killing are activated subsequently following recruitment and phosphorylation of Zap70, activation of phospholipase C, stimulation of phosphatidylinositol 3-kinase, increase in intercellular Ca^{++} levels and activation and translocation of several transcription factors (reviewed by Lanier 1998).

Cytotoxicity: Mechanism and Regulation

The NK cell-target interaction induces elevations in intercellular Ca^{++} concentrations, which in turn stimulates release of granules containing perforin and several granzymes. Perforin is a cytolytic protein that polymerizes to form membrane pores on the target cell (Lowin et al.1995). It has homology with complement-9, which also forms similar pores on the cell membranes (Uellner et al. 1997). Granzymes belong to a large family of serine proteases including granzymes A-G, metase-1, and triptase-2 which activate apoptosis when transported into the cytoplasm of the target cell (Smyth et al. 1996, Kam et al. 2000).

The mature perforin molecule is produced from a premature form of the protein through enzymatic processing (Uellner et al. 1997). The perforin is stored in lytic granules that are acidic and contains proteoglycans (reviewed by Griffiths and Argon 1995). At low pH, perforin is associated with proteoglycans (Mason et al. 1990). The binding and polymerization of perforin on the cell membrane is a Ca++ dependent process (Masson et al. 1990). It has been suggested that release of perforin induces an increase in intracellular [Ca^{++}] and increases pH, which stimulates disassociation of perforin from proteoglycans (Masson et al. 1990). Free perforin then binds and polymerizes on the cell membrane to form pores (Masson et al 1990), which allow entry of water, salt and granzymes to enter into the cell (Kagi et al. 1994). The target cell is destroyed by osmotic lysis and apoptosis is mediated by granzymes (Darmon et al. 1996). Presence of perforin and granzymes is crucial for NK cytotoxicity, because perforin knockout mice cannot exert cytotoxic responses (Kagi et al. 1994, Simon et al. 1997). Furthermore, mast cells transfected with the perforin gene but not with granzyme genes can lyse target cells but not efficiently (Nakajima et al. 1995). In contrast, the mast cells that were transfected with genes for both perforin and granzyme lysed target cells as efficiently as NK cells (Nakajima et al. 1995). The importance and function of perforin for NK cell function has also been demonstrated by treating NK cells with molecules that inhibit acidification of lytic granules and perforin processing such as concanomycin-A (CMA), which is an inhibitor of the proton pump that maintains acidity in endosomes; ammonium chloride (Kataoka et al. 1994, Kataoka et al. 1996), which increases pH of endosomal compartments; leupeptin (Hudig et al. 1984), which is a reversible inhibitor of

serine and cysteine proteases; and pepstatin-A (Uellner et al. 1997), which is an inhibitor of aspartic proteases.

Regulation by Cytokines

Natural killer cell responses can be triggered by cytokines such as IL-2, IL-12, IL-15, IL-10, IFNα/β and TGFβ of the innate and adaptive immune system (reviewed by Biron et al. 1999). Interleukin-2 promotes proliferation, lytic activity and development of NK cells into LAK cells (Grimme et al. 1982, Trinhieri 1989). Lymphokine activate killer cells are lytic cells that can lyse a wide variety of target cells including cells without expression of classical MHC class I (Grimme et al. 1982). Like IL-2, IL-15 is an efficient activator of NK cell cytotoxic activity (Carson et al. 1994) and is important for development and differentiation of NK cells from their precursors (Doherty et al. 1996). Interleukin-15 activates human NK cells using β and γ subunits of the IL-2 receptor (IL-2R) and promotes NK cell production of IFN-γ as well as IL-12 (Carson et al. 1994). In addition to IL2Rβ and γ subunits, IL-15 binds an α chain specific for the IL15 receptor, which is distinct from α chain of IL-2R (Giri et al. 1995).

Interleukin-12 is another potent inducer of NK function such as production of IFN-γ (Chan et al. 1991) and NK cytotoxicity (Lehman et al. 2001). Cytotoxicity of NK cells can be enhanced by type-I interferons such as IFNα and β released from infected cells (Orange and Biron 1996). Interleukin-10 and TGF-β are negative regulators of NK function. For example, while IL-10 can inhibit NK cell IL-12 production, TGF-β inhibits production of IL-12 and IFN-γ, proliferation and cytotoxicity of NK cells (reviewed in Biron et al. 1999).

Role of NK Cells

Antiviral defense. One of the most important roles of NK cells in host defense is antiviral defense. Down-regulation of the classical MHC class I molecules is an important strategy that some viruses utilize to evade host T cell immune responses (Lanier 1998). However, down-regulation of MHC class I expression makes virus-infected cells potential NK cell targets (Trinchieri 1989). Natural killer cells provide protection against various viruses such as herpes, influenza, adenoviruses, and human immunodeficiency virus (reviewed in Biron et al. 1999). Cytotoxicity of NK cells is enhanced during various viral infections (Trinchieri et al. 1989). The production of IFN-α, IFN-β, IL-15 and IL-12 from virus-infected cells stimulates IFN-γ production and activates NK lysis (reviewed by Biron 1999, Azimi et al. 2000, Ahmad et al. 2001).

Anti-Tumor Activity. NK cells are also very important for clearance of various tumors. Because, expression of classical MHC class I molecules is down-regulated in various tumor cells, they become susceptible to nonrestricted NK cell lysis (Trinchieri 1989, reviewed in Lanier 1998).

Antibody-dependent cell-mediated cytotoxicity (ADCC). One of the most studied activatory NK membrane receptors, CD16 (FcγRIII), is a low affinity receptor for IgG. The CD16 on NK cells mediates ADCC, which is killing of antibody-coated target cells by cells with Fc receptors that recognize the Fc region of the bound antibody (reviewed in Lanier 1998). Upon Fc receptor-Fc interaction, NK cells are activated through positive signals from the Fc receptor, which results in secretion of cytokines and lysis of the target cell (reviewed by Lanier 1998).

Cytokine Secretion. Natural killer cells secrete various cytokines and chemokines upon stimulation. For example, NK cells produce IFN-γ, IL-2, tumor necrosis factor (TNF) -α, and chemokines such as macrophage inhibitory protein-1 and -2α during viral infections (reviewed in Biron et al. 1999). Interferon-γ is a potent inducer of Th1 responses, (O'Neil et al. 1999). Like IL-2, tumor necrosis factor, promotes development of NK cells into LAK cells (Chaudhury and Knapp 2001).

NK Cells During Pregnancy

MHC Expression on Trophoblast

The major histocompatibility complex of genes encodes two major families of MHC cell surface proteins, MHC class I and class II, that play critical roles in the determination of self versus nonself by the immune system (Rossini et al. 1999). MHC class II proteins, which are typically expressed on immune cells, (Fernandez et al. 1999), are absent on the trophoblast of both mice and humans (Billington 1988). Expression of MHC class I proteins on the trophoblast, which are also largely absent, is more complicated. Three sets of MHC class I genes in human (HLA-A, -B, and -C) (Messer et al. 1992) and mouse (H-2–K, –D, and –L) (Klein et al. 1981) are referred to as MHC class I genes or MHC class Ia. These genes are highly genetically polymorphic and expressed on almost all nucleated cells (Chaudhury and Knapp 2001). In addition, there are many other MHC class I genes called nonclassical MHC class I or MHC class Ib that display very low polymorphism and are expressed in a limited repertoire of cells (Messer et al. 1992). These include HLA-E, -F, and -G in humans and Q (Qa-1, Qa-2, $Q5^k$, and Q10), T (T1a), and M in mice (reviewed by Fernandez et al 1999).

In humans, expression of HLA class Ia expression has been detected on sperm cells and mature spermatozoa (Fernandez et al. 1999). However, membrane expression of HLA class Ia (especially, HLA-A and -B) has not been observed on the preimplantation embryo (Fernandez et al. 1999) or any trophoblast subpopulation (Billington 1988, Choudhury and Knapp 2001). In contrast, class Ia proteins have been found in villous cytotrophoblast cells that cover the villous core (Guillaudeux et al. 1995). There is no expression on syncytiotrophoblast, which lines the intervillous space (Billington 1988, Choudhury and Knapp 2001). Unlike HLA- A and –B, transcripts and proteins for HLA-C have been found in different types of cytotrophoblast cells. This includes interstitial trophoblast that is interposed between the maternal cells of the decidua basalis and the large and multinucleated cells trophoblastic giant cells that represent the end point of invading trophoblast cells (King et al. 200a, Choudhury and Knapp 2001).

In mice, there are contradictory reports regarding expression of HLA class Ia. The MHC class Ia has been detected on mature spermatozoa and female gametes (Fernandez et al. 1999). Membrane expression of MHC class I ($H\text{-}2D^b$) has been detected in preimplantation embryos and blastocysts (Fernandez et al. 1999). Except for HLA-C, classical MHC class I expression is diminished or lost on placental cells after formation of the definitive placenta especially for cells having intimate contact with maternal blood such as syncytiotrophoblast and interstitial trophoblast (Fernandez et al. 1999).

The most studied nonclassical MHC molecule in humans is HLA-G. Expression occurs in blastocysts and earlier stage preimplantation embryos. Both HLA-G and -E are expressed during the first trimester in extravillous trophoblast, endovascular and interstitial trophoblast and trophoblastic giant cells (Kovats et al. 1990, Bulmer et al

1991, Chumbley et al. 1994, Hutter et al. 1996, King et al. 2000a, King et al. 2000b). Expression of HLA-E on the cell surface depends on the presence of signal peptides derived from certain HLA class I molecules such as HLA-G and C (Braud and McMichael 1997). HLA-G like molecules can be found at the maternal-fetal interface in other species such as the rhesus monkey in which Mamu-AG shares characteristics with HLA-G (Slukvin et al. 1998). In addition an H-2-linked MHC sequence has been found in mouse blastocysts that has been suggested to be a homolog of HLA-G (Sipes et al. 1996).

Lysis of Trophoblast by NK Cells

Functionally, NK cells can lyse various target cells (Trinchieri 1989). Trophoblast cells are potential NK cell targets because of down-regulation of classical MHC class I molecules on these cells. Moreover, NK cells have been found in uterine tissues of humans (Kiso et al. 1992, Ho et al. 1996), mice (Parr et al. 1990, Croy et al. 1997) and pigs (Yu et al. 1994). Spontaneous abortions seen in the DBA/2xCBA/J abortion-prone mouse model are associated with infiltration of NK cells into implantation sites (Gendron et al. 1990, Duclos et al. 1994). Also activation of NK cells with polyI•polyC leads to increased abortion in mice (Kinsky et al. 1990, Liu and Hansen 1993). However, despite the lack of MHC class I antigen expression on the trophoblast, trophoblasts from human (King and Loke 1990) and mouse (Zuckermann and Head 1987) are resistant to NK cell lysis. In contrast, the porcine trophoblast was susceptible to NK lysis even in the absence of IL-2 stimulation (Yu et al. 1994). Lysis of the trophoblast by IL-2 activated NK cells occurs in human, mice and sheep (Drake and Head 1989, King and Loke 1990, Segerson and Gunsett 1994).

These results support the assumptions that NK cells in the uterus can recognize the trophoblast but that trophoblast cells employ a strategy to inhibit NK cell activation following recognition. This inhibition, in turn, can be overridden by cytokine activation to activate NK cells. One candidate for an NK-inhibitory molecule on the trophoblast is the nonclassical MHC class I molecules that are expressed on human and mouse trophoblast cells (Choudhury and Knapp 2000, Fernandez et al. 1999, King et al. 2000a). Of these nonclassical MHC class I molecules, the most studied is HLA-G in humans. Recent studies have demonstrated that HLA-G inhibits NK cell lysis (Rouas-Ferris et al. 1997, Carosella et al. 1999). In addition, HLA-G is recognized by NK cell receptors such as CD94/NKG2A, KIR, Ly49 and ILT-2 (Colonna et al. 1997, Cosman et al. 1997, Cantoni et al. 1998).

There are several recent reports that question the role of HLA-G or other nonclassical MHC class I molecules in protection of the trophoblast. For example, trophoblast-derived JEG3 cells that were treated with acid to remove surface HLA-G were not susceptible to NK lysis (Avril et al. 1999), suggesting that there are other NK inhibitory molecules other than HLA-G (Avril et al. 1999). Also HLA-G tetrameric complexes did not bind to peripheral blood NK cells (King et al. 2000a). Immunoneutralization of HLA-G, CD94/NKG2, KIR and ILT2 did not cause target lysis (King et al. 2000a). Additionally, NK killing of target cells that express HLA-E is inhibited only when CD94/NKG2A is expressed at high levels on decidual NK cells; immunoneutralization of CD94 resulted in lysis of the target cells (King et al. 2000b).

Changes in NK Cell Biology during Pregnancy

Peripheral Blood. In humans, the majority of peripheral blood NK cells have a

phenotype of CD56 dim^{+}, CD16^{+} CD3- which distinguishes them from uterine NK cells, which are CD56 bright $^{+}$, CD16^{-} CD3^{-} (Whitelaw and Croy 1996). A relatively small portion of peripheral NK cells has a phenotype of either CD56bright^{+} CD16^{-} CD3^{-} or CD56bright^{+} CD16^{-} CD3^{+} (Lanier et al. 1986).

Unlike uterine NK cells, pregnancy status does not cause significant changes in peripheral blood NK cells. However, changes can be observed during abnormal pregnancies in humans. For example, the percentage of CD56+ CD3+ NK cells in the peripheral blood of patients with a history of recurrent spontaneous abortion (RSA) was less than that in non-pregnant women or pregnant women (Yahata et al. 1998). Immunization with paternal lymphocytes increased the population of CD56+ CD3+ NK cells in the peripheral blood of women with RSA (Kwak et al. 1998). Pregnant women with a history of RSA who subsequently experienced a successful pregnancy outcome had significantly lower CD16 expression on NK cells compared to women who experienced an additional abortion (Jerzak et al. 1998). In another study, levels of NK cytotoxicity and numbers of both single CD56+ and double CD56+ CD16+ cells were similar between nonpregnant women with a history of RSA and normal nonpregnant women. During pregnancy, however, women with RSA had higher numbers of double CD56+ CD16+ cells and increased NK cell cytotoxicity (Emmer et al. 2000). A recent study demonstrated that there was a significant increase in CD69 expression on CD56+ NK cells in women with a history of RSA as compared to that of normal controls (Ntrivalas et al. 20001). In addition, expression of the NK cell inhibitory receptor, CD94, was significantly decreased in women with RSA in comparison with values for normal women (Ntrivalas et al. 2001).

Uterus. The presence, distribution and characterization of uterine leukocytes in the uterus have been studied most extensively in mice, human and pigs. In these species, NK cells are one of the major lymphocytes in the uterine leukocyte population. In general, uterine NK cells are characterized as large granular lymphocytes (LGL). Like peripheral NK cells, uterine NK cells in mice (Whitelaw and Croy 1996), humans (King and Loke 1996), and pigs (Yu et al. 1993, Yu et al. 1993, Engelhard and King, 1995) express lytic molecules and exert lytic activity against several NK cell targets such as K562 cells (King and Loke 1990, Parr 1991, Yu et al. 1993). Furthermore, human uterine NK cells produce colony stimulating factor (CSF) -1, GM-CSF, IFN-γ, TNF-α and leukemia inhibitory factor (LIF) (reviewed in Ashkar and Croy 2001). Mouse uterine NK cells secrete CSF-1, GM-CSF, IFN-γ, IL-1, LIF, TNF-α, and vascular endothelial growth factor (VEGF) (reviewed in Ashkar and Croy 2001).

Mouse uterine NK cells have been characterized as CD45+, Thy-1+ and asialo-GM1+ (Mukhtar et al. 1989, Redline and Liu 1989) while human uterine NK cells are $CD56^{bright}$+, CD16-, CD2+ and CD3- (King and Loke 1991, Saito et al. 1993). Uterine CD56+ NK cells in human were CD45RO+ indicating that they are activated and some were also γδ T+ (Mincheva-Nilson et al. 1994).

In the mouse, uterine NK cells in the virgin uterus and prior to implantation are very low in number and distributed throughout the uterus (Parr et al. 1991, Kiso et al. 1992, Delgado et al. 1996). Numbers of uterine NK cells increase during decidual development and eventually become concentrated in a specific region in at each implantation site called the metrial triangle. The metrial triangle a region between fibers of circular smooth muscle of the uterus, where a structure called the metrial gland is formed by Day

8 of pregnancy (Stewart 1994, Bulmer et al. 1987, Peel 1989, Croy and Kiso 1993). The uterine NK cells in metrial glands are also called granulated metrial gland (GMG) cells (Peel 1989). The number of mouse uterine NK cells increase during midgestation (Days 7-12) in decidua (Askar and Croy 2001). The GMG cells display degenerative changes later in pregnancy and disappear near parturition (Peel and Stewart 1979, Peel 1989).

As for the mouse, human uterine NK cells have a morphology characteristic of large granular lymphocytes (LGL) and exist in the nonpregnant uterus. Uterine NK cells proliferate and increase in number during the secretory stage of the menstrual cycle (King et al. 1989, Starkey et al. 1991). Uterine NK cells are present throughout the decidua and numbers peak at weeks 6-12 of pregnancy, after which time numbers decline (Bulmer and Sunderland 1984, King et al. 1989, King et al. 1991, Starkey et al. 1991). In pigs, uterine NK cells are high in number during early pregnancy compared with cyclic pigs (Engelhardt and King 1995, Engelhardt and King 1996). In cyclic pigs, uterine lymphocytes are located in the luminal epithelium but also populate the stroma during pregnancy (Engelhardt and King 1996). Natural killer cell activity was observed from Day 10-20 of pregnancy and disappeared by Day 30 of gestation (Croy et al 1987, Croy et al. 1988, Yu et al. 1993). Unlike mice and humans, there was no uterine NK cell activity in pseudopregnant pigs (Croy end King 1987, Croy et al. 1988, Yu et al. 1993).

Change in numbers of uterine NK cells during late pregnancy suggests that uterine NK cells are regulated by ovarian hormones and placental factors. In nonpregnant women, uterine NK cells are in greatest number during the secretory phase of the menstrual cycle (King et al. 1989, King and Loke 1991, Starkey et al. 1991). These results suggest that uterine NK cells depend on progesterone. An increase in expression of progesterone

receptors on NK cells after early gestation in mouse (Whitelaw and Croy 1996) may also imply progesterone regulation. There is also a correlation between higher levels of estrogen receptor and increased numbers of uterine NK in cycling mice (Head et al. 1994, Whitelaw and Croy 1996) which suggest estradiol is involved regulation of NK cells. Unlike humans and mice, NK activity in pigs is dependent on the presence of the conceptus, because uterine NK cells were not detected in pseudopregnant pigs (Croy and King 1987, Croy et al. 1988, Yu et al. 1993).

Regulation of NK Cells during Pregnancy by Cytokines

Various cytokines are expressed at the maternal-fetal interface in humans and mice (Saito 2000). It has been suggested that these cytokines produced by the placenta and endometrial lymphocytes regulate cell-mediated responses of NK cells to maintain pregnancy (Clark et al. 1999). For example, while IFN-α/β, IL-6, IL-12 and IL-15 could activate uterine NK cell functions such as cytokine secretion and cytotoxicity, other cytokines such as TGF-β2, LIF, IL-10 may suppress NK cell cytotoxicity (reviewed by Chaudhury and Knap, 2001).

In the DBA/2xCBA/J abortion-prone mouse, depletion γδ T cells that produce TGF-β increased abortion rates (Arck et al. 1997). Similarly, endometrial cells from cyclic and early pregnant (Days 16-18) cows suppressed proliferation of lymphocytes and suppressive activity was associated with TGF-β (Shipp and Segerson 1998). Thus, endometrial suppressor cells may contribute to immunoregulation of uterine NK cell functions during pregnancy.

Other cytokines such as IL-10, GM-CSF and IL-3 reduced fetal resorptions in DBA/2xCBA/J mice (Chaouat et al. 1990). In humans, LIF, IL-4 and IL-10 were found

in higher amounts in the decidua during normal pregnancy as compared to decidua from women with RSA (reviewed by Chaudhury and Knap 2001). Thus, these cytokines may suppress function of uterine NK cells. Another cytokine, IFN-γ can increase expression of nonclassical MHC class I molecules such as HLA-A on trophoblast cells (Grabowska et al. 1990, King and Loke 1993), suggesting that it may inhibit LAK cell cytotoxicity directed against trophoblast.

Proposed Functions of Uterine NK Cells during Pregnancy

Anti-trophoblast Activity. Both peripheral and uterine NK cells in mice, humans and pigs express lytic molecules and exert lysis against several NK cell targets (King and Loke 1990, Parr 1990, Parr 1991, Yu et al. 1994). In mice and humans, killing of the trophoblast requires activation of NK cells by IL-2 stimulation (Drake and Head 1989, Croy et al 1991). Thus, uterine NK cells can kill the trophoblast when they are transformed into LAK cells (Drake and Head 1989). In mice, GMG cells were found preferentially associated with degenerating trophoblast (Stewart 1990, Stewart 1991) suggesting that uterine NK cells can kill the trophoblast to limit invasion into the uterine endometrium. Furthermore, in mice, the majority of uterine NK cells remaining in the uterus at parturition were accumulated along the basal aspect of placenta (Delgado et al. 1996), suggesting that lytic activity of uterine NK cells might favor the parturition process.

Regulation of Implantation, Modification of Maternal Vasculature and Placental Growth. It has been proposed that uterine NK cells perform functions that are associated with successful pregnancy (Croy et al 1996). This assumption is supported by recent studies using transgenic mice such as TgE26, $p56^{lck-/lck-}$/Il-2Rβ$^{-/-}$, IL-2Rγ$^{-/-}$ and RAG-2$^{-/-}$/

$\gamma_c^{-/-}$ (Wang et al. 1994, Guimond et al. 1996, Guimond et al. 1997, Ashkar et al. 2000, Greenwood et al. 2000). In general, these uterine NK deficient mice displayed reproductive compromise characterized by small embryo size, decidual and placental disintegrity and lack of vascular remodeling and development (Guimond et al. 1997, Ashkar et al. 2000, Greenwood et al. 2000). Reconstitution of RAG-$2^{-/-}$/ $\gamma_c^{-/-}$ mice (NK-, T-, B-) with bone marrow from SCID mice (NK+, T-, B-) resulted in normal decidual development and vascular remodeling (Ashkar and Croy 2001).

Expression of IFN-γ is diminished in implantation sites in Tge26 (NK-, T-) (Wang et al. 1994) and RAG-$2^{-/-}$/ $\gamma_c^{-/-}$ mice but not in normal and SCID mice (Ashkar and Croy 2001) Thus, it has been proposed that loss of IFN-γ from uterine NK cells was the cause of reproductive compromise seen in TgE26 and RAG-$2^{-/-}$/ $\gamma_c^{-/-}$ mice (Ashkar and Croy 2001). Recent studies on transgenic mice with phenotypes of IFN$\gamma^{-/-}$, IFN-γRα or Stat-$1^{-/-}$ showed implantation site defects that were similar to those observed in uterine NK deficient mice strains (Ashkar and Croy 1999). Thus, these studies shows that NK cells are beneficial for pregnancy by mediating tissue reorganization and vascular formation through secretion of IFN-γ However, another IFN-γ deficient mouse strain did not show similar defects (Hunt et al. 2000).

Several other NK cell-derived cytokines, such as G–CSF, GM-CSF, and LIF (Croy et al. 1991, Saito et al. 1993) have also been proposed to regulate implantation and trophoblast differentiation. For example, LIF has been shown to be required for implantation because blastocysts were not able to implant in mice that lack LIF (Croy et al. 1991, Stewart et al. 1992). Furthermore, CSF can induce trophoblast differentiation and human chorionic gonadotrophin production (Croy et al. 1991, Saito et al. 1993) and

GM-CSF can promote formation of trophoblast giant cells (Drake and Head 1994, Jokhi et al. 1994). In both mice and rats, uterine NK cells were the main source of inducible nitric oxide synthase, (iNOS), which produces nitric oxide (NO) (Hunt et al. 1997). Interestingly, iNOS deficient mice had abnormal blood vessels in decidua (Hunt et al 2000), suggesting that uterine NK cells can promote development of uterine arteries by production of NO.

Pregnancy Immunobiology of the Sheep

Placentation

The sheep placenta is a synepitheliochorial type placenta. The chorionic villi from cotyledonary extraembryonic membranes penetrate into caruncules of maternal endometrium to form combined structures called placentomes. The chorionic binucleated cells fuse with uterine epithelium to form a maternal-fetal syncytium (Wooding 1982). The binucleated cells are directly involved in the modification of the uterine epithelium to produce and deliver protein and steroid hormones such as placental lactogen (Wooding 1992). The caruncular endometrium is devoid of uterine glands. In the interplacentomal regions, chorionic membrane and uterine epithelium interact without interlocking microvilli (Perry 1981). Unlike caruncules, the interplacentomal endometrium is glandular (Perry 1981).

MHC Expression at the Maternal-Fetal Interface

Gogolin-Ewens et al. (1989) have demonstrated that MHC class I molecules are present on interplacentomal and placentomal uterine epithelial cells before syncytium formation at day 16-18 of gestation. In contrast, MHC class I expression is not found on any fetally-derived placental tissues between days 9 and 125 of pregnancy (Gogolin-Ewens et al 1989). Similarly, MHC class II molecules were not detected on fetally-

derived placental tissues throughout pregnancy (Gogolin-Ewens et al. 1989). Thus, lack of MHC class I expression on the sheep trophoblast cells may protect them from T cell-mediated responses against paternal antigens but also makes the trophoblast a potential target for NK cells. Indeed, NK-like lytic function of sheep PBL has been demonstrated against certain target cells such as K562, YAC-1 (Liu and Hansen 1993, Tuo et al. 1993). Peri-attachment sheep conceptuses were lysed by IL-2 activated PBL (Segerson and Gunsett 1994). Thus, NK-like cells probably exist in sheep and their cytotoxic function must be regulated during pregnancy to protect conceptus.

Uterine Lymphocytes during the Estrous Cycle and Pregnancy

Lymphocytes in the sheep endometrium are almost exclusively localized in the epithelium or in the stroma immediately adjacent to the epithelium (Gogolin-Ewens et al. 1989, Gottshall and Hansen 1992, Majewski et al. 2001). Three intraepithelial lymphocyte (IEL) subpopulations have been described: CD8 + CD45R- γδ TCR+ cells, which comprise about 50% of IEL during the estrous cycle; CD8+ CD45R+ γδ TCR- cells, about 25 % of IEL, and CD8+ CD45R+ γδ TCR+ cells, about 25 % of IEL (Meeusen et al. 1993). In contrast, CD4+ T helper cells or B cells have not been found in endometrium during estrous cycle or pregnancy (Gogolin-Ewens et al. 1989).

During pregnancy, there are fewer T lymphocytes in the placentomes than in interplacentomal regions (Gogolin-Ewens et al. 1989), suggesting that maternal immune responses are regulated differently in these two distinct regions. The number of nongranulated lymphocytes decreases in interplacentomal epithelium as pregnancy proceeds (Lee et al. 1992). In contrast, the number of granulated CD8+ CD45R+ γδ TCR+ cells in the luminal epithelium increases in late pregnancy (Meeusen et al. 1993).

There is also an increase in granularity of these cells as pregnancy proceeds (Meeusen et al. 1993). Studies on unilaterally-pregnant ewes in which pregnancy is limited to one uterine horn showed increased expression of activation markers such as CD25 and CD44 on γδ T+ lymphocytes from the luminal epithelium of the pregnant uterine horn as compared to those from the nonpregnant uterine horn or from cyclic ewes (Liu et al. 1997). These results suggest that γδ T+ cells are activated by the local presence of conceptus during pregnancy. However, as compared to ovaryectomized ewes, the increase in numbers of γδ T cells in luminal epithelium occurred in both pregnant and nonpregnant uterine horns (Majewski et al. 2001).

It is likely that the CD8+ CD45R- γδ TCR-and CD8+ CD45R+ γδ TCR+ populations are αβ TCR+ cells. As such, these cells presumably can exert MHC I Class-restricted lysis against target cells such as virus-infected or aberrant epithelial cells. The function of the CD8+ CD45R+ γδ TCR+ cells is less clear. This cell population expresses the cytolytic molecule, perforin (Fox and Meeusen 1999). Perhaps, these cells may be analogous to NK T cells described in other species (Whitelaw and Croy 1996). In addition, the CD8+ CD45R+ γδ TCR+ cells may act as uterine suppressor cells as in the mouse, where uterine γδ T+ cells produce TGF-β and can inhibit other lymphocyte populations (Suzuki et al. 1995). They may also secrete cytokines to enhance embryonic development, as has been proposed for uterine γδ TCR+ cells in mice (Saito et al. 1993) and humans (King and Loke 1991).

Unlike most species examined, the existence of NK cells in ruminants is questionable. There is no definitive report regarding existence of the NK cells in these species. In contrast to NK cells in human and mouse, sheep PBL cannot lyse traditional NK cell

targets in a 4 h chromium release assay. Even after 20 h lysis, sheep PBL exert low levels of lysis against NK targets such as K562 (Segerson and Gunsett 1989) and YAC-1 cells (Liu and Hansen 1993). In addition, the only NK-reactive antibody that has been reported to react with sheep and cattle is anti-FAM, which is expressed in mice and human NK cells (Evans et al. 1988, Harris et al. 1993). It has been reported that ~12% of sheep lymphocytes reacted with this antibody (Evans et al. 1988, Harris et al. 1993).

Major Immunosuppressive Molecules at the Maternal-Fetal Interface

Numerous secretory molecules of the trophoblast and endometrium have been implicated in immunoregulation of immune function in sheep. Together these molecules create an environment at the maternal-fetal interface that inhibits lymphocyte responses against the conceptus. The characteristics of these molecules are summarized in this section.

Progesterone. Progesterone is a key hormone of pregnancy required for establishment and maintenance of pregnancy. In sheep, progesterone is secreted from the corpus luteum (Casida and Warwick 1945, Beal et al 1986). After Day 50 the corpus luteum is not needed for the maintenance of pregnancy (Casida and Warwick 1945, Beal et al 1986) and progesterone is produced by placental cells in sufficient quantity to maintain pregnancy. Progesterone plays a critical role for the regulation of uterine immune responses. For example, progesterone decreased numbers of MHC class II+ and CD45+ cells in sheep endometrium (Gottshall and Hansen 1992), prolonged survival of skin grafts placed in the uterus of sheep (Hansen et al. 1986), and enhanced survival of xenogeneic cells implanted into the sheep uterus (Majewski and Hansen, in press). At very high concentrations (10^{-6} to 10^{-5} M), progesterone directly inhibits mitogen-induced proliferation of lymphocytes (Staples et al. 1983, Low and Hansen 1988, Monterroso and

Hansen 1993). These concentrations are higher than those causing changes in skin graft survival (10^{-7}M). Thus, while direct immunosuppressive effects of progesterone may be important after Day 50 of pregnancy, when placental progesterone synthesis is established, it is likely that other effects of progesterone are exerted indirectly. In particular, progesterone can induce immunosuppressive activity in uterine secretions (Hansen et al. 1986, Hansen and Skopets 1992). Much of this induced activity is due to the molecule ovine uterine serpin (OvUS), which is described further.

Interferon-τ. In ruminants, maternal recognition of pregnancy requires production of a specific type I interferon called interferon-τ (Roberts et al 1999). Interferon-τ genes are found in various ruminant species such as sheep, cow, goat, musk, oxen, and giraffe (Imakawa et al. 1987, Roberts et al. 1999). Amino acid sequencing and molecular cloning revealed that IFN-τ is a member of the type I interferon family consisting of IFN-α, β, δ and ϖ (Imakawa et al. 1987, Roberts et al. 1999). Genes for IFN-τ probably arose from IFN-ω about 36 million years ago (reviewed by Demmers et al. 2001).

In sheep and cattle, there are multiple genes for IFN-τ and several of these genes are transcribed during early pregnancy (Ealy et al. 1998, Martal et al. 1998). In the sheep, recombinant proteins for some of these IFN-τ variants have been expressed. Among these are variants termed IFN-τ4, IFN-τ6d, IFN-τ2c and IFN-τ11 (Roberts et al. 1997, Ealy et al 1998). These proteins have high homology (>70%) in their primary amino acid sequences (Imakawa et al. 1987, Leaman et al. 1992, Nephew et al. 1993). Relative abundance of expressed proteins for IFN- τ4, IFN-τ6d, and IFN-τ2c in preimplantation conceptus is apparently equal (Winkelman et al. 1999). Ovine IFN-τ4 is the most potent antiviral IFN-τ (Ealy et al. 1998, Winkelman et al. 1999) and more effective at inhibiting

growth of human Daudi cells than IFN-τ6d or IFN-τ11 (Ealy et al. 1998). Furthermore, OvIFN-τ4 is also the most potent antiluteolytic molecule among all OvIFN-τ variants tested (Ealy et al. 1998).

In sheep, interferon-τ is expressed by pre-implantation trophoblast between days 12-21 of pregnancy (Godkin et al. 1982, Helmer et al. 1987, Martal et al 1998). Although not observed by all investigators (de Moraes et al. 1997), it has been reported that GM-CSF increases IFN-τ secretion (Imakawa et al. 1993). The major physiological role of IFN-τ is to block regression of the corpus luteum by inhibiting pulsatile secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and allowing establishment of pregnancy (Bazer et al. 1997, Roberts et al. 1999). In addition, OvIFN-τ can induce expression of genes that are normally regulated by other type I IFNs, such as 2', 5' oligoadenylate synthase (Johnson et al 2001), granulocyte chemotactic protein-2 (Demmers et al. 2001), ubiquitin cross-reactive protein (Johnson et al 2001), Mx protein (Ott et al. 1998), and interferon regulatory factor-1 and -2 (Schmitt et al. 1993). Interferon-τ and IFN-α appear to bind the same type I IFN receptor (Pontzer et al 1994). Both can inhibit $PGF_{2\alpha}$ secretion in vitro (reviewed by Martal et al 1998). Although they are similar, IFN-τ is much less toxic than IFN-α (Pontzer et al. 1991, Pontzer et al. 1997), suggesting that IFN-τ can be a potential therapeutic agent for treatments of some viral diseases.

Interferon-τ has immunoregulatory properties characteristic of other type I interferons. For example, IFN-τ is antiviral (reviewed by Martal et al 1998) and immunomodulatory. It enhances NK cell lysis towards K562 and HeLa cells in vitro (Tuo et al. 1993), increases expression of IFN-γ and IL-4 in bovine lymphocytes and cultured CD4+ cells (Tuo et al. 1999a) and reduces the proliferative responses of lymphocytes to IL-2

(Niwano et al. 1989). It also inhibits proliferation of several nonlymphoid cells (Ponzer et al. 1991, Kamwanja and Hansen 1993) and mitogen-induced proliferation of lymphocytes (Newton et al. 1989, Nivano et al. 1989, Pontzer et al. 1991, Skopets et al. 1992). Tuo et al. (1998) reported that the percentages of CD5+, CD4+ and γδ T+ cells decreased after IFN-τ injection into mice. In addition, IFN-τ reduced the subpopulation of WC1+ CD2- CD6- CD8+ γδ T+ cells and increased the numbers of subpopulation of WC1- CD2+ CD6+ CD8+ γδ T+ cells in antigen stimulated cultures of bovine lymphocytes (Tuo et al. 1999b). Therefore, the regulatory effect of IFN-τ may depend on cell type.

The immunoregulatory role of IFN-τ at the maternal-fetal interface is unclear. There is a decline in overall number of luminal endometrial lymphocytes at day 20 of pregnancy (Staples et al. 1983). However, Leung et al. (2000) did not find any change in CD4+ lymphocyte populations in the endometrium of pregnant cows at the time of IFN-τ production as compared to cycling cows. That IFN-τ may have an important immunoregulatory function during pregnancy is evidenced by the finding that IFN-τ treatment reduced fetal resorptions in the DBA/2xCBA/J mouse model, and increased secretion of IL3, IL4 and IL10 in endometrium (Chaouat et al. 1995). In addition, IFN-τ inhibited development of experimental allergic encephalomyelitis (EAE) (Soos et al. 1995, Soos et al. 1997) by decreasing TNF-α secretion (Soos et al. 1995) and autoantibody formation (Mujtaba et al. 1998). In the EAE model, IFN-τ stimulated IL10 and TGF-β synthesis from CD4 + cells to suppress function of myelin basic protein specific T cells (Mujtaba et al. 1997).

Prostaglandin E2. Prostaglandin E_2 is secreted from conceptus and endometrium throughout pregnancy (Challis et al. 1982). This prostaglandin is a potent immunosuppressant that can inhibit PHA-induced proliferation lymphocytes and mixed lymphocyte reaction (MLR) at concentrations as low as 10^{-8} (Low and Hansen 1988). Therefore it is possible that during sheep pregnancy PGE_2 may contribute to immunoregulation of immune responses at the maternal-fetal interface.

Placental Lactogen. Placental lactogen is secreted from the placenta as early as Day 14 to 16 in ruminants (Martal and Djiane 1977) and peaks between Days 60 to term in sheep (Kappes et al. 1992). Human placental lactogen inhibits IL-2 secretion in lymphocytes (Schafer et al. 1992).

Biology of Ovine Uterine Serpin

Endometrial Secretion of Ovine Uterine Serpin

Ovine uterine serpin (OvUS) is the most abundant protein in uterine secretions of sheep after day 20 of pregnancy (Bazer et al. 1979, Moffatt et al. 1987, Ing et al. 1989, Stephenson et al. 1989, Leslie and Hansen 1991). The uteri of pig and cattle also secrete similar glycoproteins during pregnancy (Malathy et al. 1990, Leslie and Hansen, 1991). The inferred amino acid sequences for bovine uterine serpin (BoUS-1) and OvUS exhibit about 84% sequence identity to each other but only have about 50% and 56% identity, respectively, to two distinct porcine uterine serpins (PoUS-1 and PoUS-2) (Peltier et al 2000a).

The mRNA for OvUS can be detected in endometrium as early as day 14 of pregnancy and Day 13-16 of the estrous cycle (Ing et al. 1989, Stewart et al. 2001). A recent study demonstrated that steady-state levels of OvUS mRNA in the intercaruncular

endometrium increased about 3-fold between days 20 and 60 of pregnancy, another 3-fold between days 60 and 80 of pregnancy, and then declined slightly to day 120 (Stewart et al. 2000). In contrast to reports that OvUS protein is present in endometrial glands and luminal epithelium (Ing et al. 1989, Stephenson et al. 1989), OvUS mRNA expression was restricted to the endometrial glandular epithelium (Stewart et al. 2001).

The secretion of OvUS is stimulated by progesterone (Moffat et al. 1987, Ing et al. 1989, Stephenson and Hansen 1990). Ovine uterine serpin can be detected after 4-6 days of daily progesterone injection in sheep (Ing et al. 1989). However, prolonged treatment of 14 to 30 days is required to cause a large increase in OvUS secretion (Ing et al. 1989, Leslie and Hansen 1991). Ovariectomized ewes that were administered progesterone and estradiol together had reduced mRNA for OvUS compared with ovariectomized ewes treated with progesterone only (Gray et al. 1998). It has been suggested that coadministiration of progesterone and estradiol suppress expression of progesterone receptors on glandular cells and therefore block OvUS mRNA expression induced by progesterone (Spencer et al. 1999). Furthermore, placental lactogen can also affect production of OvUS. Intrauterine infusion of placental lactogen did not alter induction of OvUS production by progesterone (Gray et al. 1998). However, administration of placental lactogen to ovariectomized ewes receiving daily injections of progesterone and IFN-τ increased steady-state levels of OvUS mRNA (Spencer et al. 1999).

Biochemical Properties

Ovine uterine serpin exists as a pair of glycoproteins with Mr of 55 and 57 kDa produced from a single 53 kDa precursor (Hansen et al. 1987a, Ing et al. 1989, Leslie and Hansen 1990). The two forms of OvUS have a similar peptide structure as determined by

peptide mapping analysis (Hansen et al. 1987b). It is suggested that the two mature forms are products of post-translational modification of a precursor protein (Hansen et al. 1987a). Since OvUS is glycosylated and has two N-linked glycosylation sites, it is possible that the large and small forms of OvUS differ in number of N-linked glycosylation sites (Ing and Roberts 1989).

Ovine uterine serpin belongs to the serpin superfamily of serine proteinase inhibitors that also contains $\alpha_1$antitrypsin, antithrombin-III and ovalbumin (Moffat et al. 1987, Ing and Roberts 1989, Malathy et al. 1990, Mathialagan and Hansen 1996). Analysis of the evolutionary history of uterine serpins indicates that the uterine serpins form a separate clade within the serpin superfamily and that uterine serpin diverged from other serpins families before the divergence of artiodactyls (Peltier 2000a). Unlike typical serpins, OvUS does not exert antiproteolytic activity to any serine proteinases (Ing and Roberts 1989) but is weakly inhibitory to aspartic proteinases such as pepsin (Mathialagan et al. 1995, Mathialagan and Hansen 1996).

Structure-function studies on OvUS have led to the conclusion that OvUS has evolved to have a tertiary structure distinct from other serpins (Peltier et al. 2000a). Unlike a typical serpin, limited proteolysis of OvUS did not result in cleavage at the putative reactive center loop, suggesting that the reactive center loop on OvUS is not exposed as in other serpins (Peltier et al. 2000b). In addition, limited proteolysis of OvUS did not affect secondary structure, thermal stability or biological activity (Peltier et al. 2000b). The preincubation of OvUS with synthetic peptides matching the putative reactive center loop did not affect structure, thermal stability, or biological activity of OvUS (Peltier et al. 2000b), although such a treatment typically leads to inactivation of serpin function.

Ovine uterine serpin can bind to an inactive group of aspartic proteinases produced by syncytiotrophoblast, heparin, pregnancy associated proteins, IgA, IgM, lymphocyte surfaces and activin (Hansen and Newton 1988, Xie et al. 1991, Xie et al.1994, Mathialagan and Hansen 1996, Liu et al. 1999, McFarlane 1999). Ovine uterine serpin and its homologue in pigs can cross the placenta (Baumbach et al. 1986, Newton et al. 1989b), suggesting that the proteins may play a role in placental transport of proteins or other molecules. Similarly, PoUS binds the iron-containing protein, uteroferrin (Baumbach et al. 1986). In addition, OvUS is present in colostrum (Hansen and Foti 1986), suggesting that it may protect proteins from pepsin digestion in the neonatal gut.

Immunosuppressive Function

Ovine uterine serpin has been shown to inhibit a wide variety of immune functions. For example, OvUS can inhibit mixed lymphocyte reaction, phytohemagglutinin (PHA), concanavalin A (ConA), and *Candida albicans* antigen induced lymphocyte proliferation (Segerson et al. 1984, Stephenson et al. 1989, Stephenson et al. 1990, Zhang and Miller 1989, Skopets and Hansen 1993, Skopets et al. 1995). OvUS inhibited polyI•polyC-induced NK lysis of cultured sheep lymphocytes and mouse splenocytes and abortion in vivo (Liu and Hansen, 1993). Ovine uterine fluid inhibited T cells-dependent antibody production in mice (Stephenson et al. 1991). Similarly, OvUS reduced antibody response to ovalbumin in ewes (Skopets et al. 1995).

However, OvUS did not exert immunosuppressive activity against pokeweed mitogen-induced lymphocyte proliferation (Skopets and Hansen 1993). It also did not inhibit costimulation of PHA-induced proliferation of lymphocytes by CD26 (Liu and Hansen 1995). Also Skopets et al. (1995) reported that OvUS did not effect skin-fold thickness-

induced by *Mycobacterium tuberculosis*. Since this bacterium activates γδ T cells and since γδ T cells increase during late pregnancy in the presence of OvUS, it is possible that OvUS may not suppress proliferation of γδ T + cells. Indeed, Peltier et al. (2000c) recently showed that OvUS could inhibit ConA induced expression of CD25 in γδ T- cells but not in γδ T+ cells, suggesting that OvUS may preferentially inhibit γδ T- cell population.

The exact mechanism by which OvUS blocks lymphocyte proliferation is unknown. Liu et al. (1999) showed that OvUS could bind lymphocytes. In addition, OvUS posses several protein kinase-C, tyrosine kinase, and cyclic adenosin monophosphate phosphorylation sites (Peltier et al. 2000a), suggesting that OvUS involves signal transduction to control T-cell activation. Also, OvUS inhibited IL-2–stimulated lymphocyte proliferation (Peltier et al. 2000c). Peltier et al. (2000c) showed that OvUS could inhibit lymphocyte proliferation by blocking the upregulation of mRNA for IL-2 receptor but not IL-2 mRNA (Peltier et al. 2000c).

Summary

During pregnancy, the maternal immune system is potentially able to recognize and interact with the allogeneic conceptus. While down-regulation of the expression of MHC class I and II molecules on the trophoblast renders the trophoblast less antigenic, this strategy also exposes the trophoblast to potential lysis by NK cells and LAK cells. The trophoblast appears to remain resistant to NK lysis because of expression of molecules that block NK cell activity but there is also evidence that trophoblast remains susceptible to LAK cells. Generation of LAK cells may be prevented by one or more of the specific immunoregulatory factors present at the maternal-fetal interface that down-regulate NK

cell function or LAK cell activation. In sheep, progesterone appears to be a major regulator of immune function and it is likely that induction of immunosuppressive molecules such as OvUS act to inhibit NK cell responses. However, not all functions exerted by NK cells are necessarily harmful to the conceptus, as recent evidence in mice indicates, and it is possible that activation of NK cells at specific times during pregnancy may be beneficial to pregnancy. In the sheep, IFN-τ is one possible signal for upregulation of NK cell function.

CHAPTER 3
LYMPHOCYTE-MEDIATED LYSIS OF SHEEP CHORION: SUSCEPTIBILITY OF CHORIONIC CELLS TO SPECIFIC AND NONSPECIFIC CYTOTOXIC LYMPHOCYTES AND PRESENCE OF CELLS IN THE ENDOMETRIUM EXHIBITING CYTOTOXICITY TOWARD NATURAL KILLER CELL TARGETS

Introduction

In allogeneic pregnancies, the conceptus is potentially susceptible to destruction by immune cells of the mother that recognizes paternally-inherited antigens on the surface of the trophoblast. While CTL specific for trophoblast can be generated in vitro under certain conditions (Toder et al. 1982, Blank et al. 1987, Parhar et al. 1989), there is little evidence for specific cell-mediated immunity against trophoblast in pregnant females (Billington et al. 1993). Indeed, murine trophoblast can be successfully transplanted across strains without its immunological rejection (Simmons and Russell 1962, Hunziker and Wegman 1986). One reason CTL may not be generated against trophoblast is that this tissue has altered expression of major histocompatibility antigens. In the human (Hutter et al. 1998) and sheep (Gogolin-Ewens et al. 1989), for example, classical MHC class I and II antigens are not expressed on trophoblast. The absence of these molecules makes trophoblast potentially susceptible to lysis by NK cells since cells are ordinarily protected from lysis by NK cells by the presence of MHC class I (Storkus et al. 1989). However, trophoblast from both mouse (Zuckermann and Head 1988, Drake and Head 1989) and human (Ferry et al. 1991, Rouas-Freiss et al. 1997, Avril et al. 1999) is resistant to NK cell lysis although IL-2 can induce killing mediated by LAK cells (Drake and Head 1989, King and Loke 1990, Avril et al. 1999). In contrast, the preimplantation

pig trophoblast is susceptible to NK lysis (Yu et al. 1994). The trophoblast can also resist lysis by specific CTL (Zuckermann and Head 1987) even when expression of MHC class I antigens is induced by interferon (Chaouat and Kolb 1985).

In ruminants, little is known about the interaction between the conceptus and cytotoxic maternal cells. In early pregnancy, the trophoblast is potentially susceptible to lysis because sheep NK and LAK cells were cytotoxic towards preattachment ovine conceptuses (Segerson and Gunsett 1994). Moreover, there is a large increase in numbers of γδ granulated T lymphocytes in the luminal epithelium at mid to late gestation (Lee et al. 1992). These cells may be cytotoxic because they are $CD8^+$, granulated, and contain mRNA for perforin and cytokines such as IFN-γ and TNF-α (Meeusen et al. 1993, Fox et al. 1998, Fox and Meeusen 1999). In unilaterally-pregnant ewes, where the conceptus is confined to one uterine horn, γδ T cells from the pregnant horn have been reported to have increased expression of activation markers, suggesting stimulation by the local presence of the conceptus (Liu et al. 1997), although this finding has been questioned (Fox et al 1998, Majewski et al. 2001).

In the present study, it was tested whether sheep trophoblast is susceptible to lysis by specific and non-specific maternal immune cells in peripheral blood, whether cultured ovine trophoblast cells are susceptible to cytotoxic lymphocytes in peripheral blood, whether PBL exert cytotoxicity against the NK target, D17 cells (Palmer et al. 1990), and if killing is enhanced by IL-2 and infection with Bovine Herpes Virus (BHV)-1, whether endometrial epithelial cells kill BHV-1 infected D17 cells are present in endometrium of the sheep and whether endometrium produces a lytic molecule, perforin.

Materials and Methods

Materials

Control mouse ascites fluid (clone NS1), sheep affinity-purified, FITC-labeled $F(ab)_2$ fragment against mouse IgG (whole molecule), and ascites fluids for mouse monoclonal antibodies against cytokeratin (clone KB-37) and vimentin (Clone V9) were purchased from Sigma Chemical (St. Louis, MO). Affinity-purified mouse monoclonal antibody that recognizes sheep MHC class I (H58A) was from WMRD (Pulman, WI). Mouse monoclonal antibody against sheep MHC class I (SBU-I) was obtained as ascites fluid from Center for Animal Biotechnology, Melbourne, Australia. Hybridoma cells producing monoclonal antibody against human perforin (clone 2d4) were a gift from Dr. G. Griffiths (Oxford University, UK). Antibody was obtained as culture supernatant from hybridoma cell cultures prepared by the Hybridoma Core Laboratory of the University of Florida Interdisciplinary Center for Biotechnology Research.

Tissue culture medium-199 (M-199), Eagle's minimal essential medium (MEM), glutamine, bovine serum albumin (BSA), penicillin-streptomycin, red cell lysis buffer, β-mercaptoethanol, trypsin-EDTA , Dulbecco's phosphate buffered saline (DPBS), and Triton X-100 were from Sigma. The $PGF_{2\alpha}$ was purchased from Upjohn-Pharmacia (Kalamazoo, MI). Human recombinant IL-2 was from Genzyme (Boston, MA). Fico-Lite-1077, normal goat serum and fetal calf serum were from Atlanta Biologicals (Norcross, GA). Horse serum was from Hyclone (Logan, UT). Fetal bovine serum was from Intergen (Purchase, NY). $Na^{51}CrO_3$ [specific activity: 571 mCi/mgCr] was from ICN. The D17 cell line (canine osteocarcinoma) and BHV-1 [TCID for 0.2 mL bovine turbinate cells, 10 d culture, $TCID50 = 10^{6.5}$) were from ATCC (Rockville, MD). The

T75 cell culture flasks were from Sarstedt (Newton, NC), 96-well Falcon culture plates and cell strainer (100 μm) were from Becton Dickinson (Franklin Lakes, NJ) and glass chamber slides were from Nalgene (Nunc, Naperville, IL). H_2O_2, paraformaldehyde, 12x75 mm polyethylene culture tubes and glass microscope slides were from Fisher Scientific (Pittsburgh, PA). The Histoscan Monoclonal Detector kit for performing immunohistochemistry was obtained from Biomeda (Foster City, CA). Embedding medium (Tissue-Tek OCT Compound) was obtained from Miles Diagnostic (Elkhart, IN).

Experimental Design for Trophoblast Lysis

Pregnancies were established in adult cycling ewes of predominantly Rambouillet genotype (n=7). Estrous cycles were synchronized by 2 injections, i.m. of 2 mg $PGF_{2\alpha}$ at 11 day intervals. Ewes were bred at estrus to fertile rams to establish pregnancy. Ewes were slaughtered by captive-bolt stunning at Day 51-91 of gestation and the placenta recovered. Trophoblast cells, prepared as described below, were labeled with ^{51}Chromium and used a target cells in a chromium release assay using PBL from the pregnant female (maternal cells) and from third-party ewes. Third-party ewes included pregnant ewes (n=3) and cyclic ewes (n=7). All animals were multiparous. Each preparation of trophoblast was tested separately using PBL from one or two third-party ewes. As a control, each preparation of PBL was also evaluated for ability to lyse D17 cells, which are a target for lysis NK-like cells. For both trophoblast and D17 cells, the killing assay was performed with unstimulated PBL and PBL cultured with IL-2 and with untreated target cells and target cells infected with BHV-1.

Experimental Design to Evaluate Presence of NK-like Cells in Endometrial Epithelium

Multiparous ewes that were predominately of Rambouillet breeding (n=7) were surgically made unilaterally pregnant to limit pregnancy in only one horn as described elsewhere (Bazer et al. 1979). Briefly, one uterine horn was ligated surgically and the ovary ipsilateral to the ligated horn was also removed. After > 30 days of recovery, estrous cycles were synchronized as described above and ewes were bred at estrus to fertile rams to establish pregnancy. Ewes were slaughtered by captive-bolt stunning at Day 140 of gestation and the pregnant uterus recovered for collection of endometrial epithelium as described below. Endometrial epithelium was also obtained from 5 cyclic ewes slaughtered on random days of the estrous cycle. Each preparation of endometrial epithelial cells was tested separately for NK-like lysis against BHV-1 infected D17 cells. In addition, tissue samples were snap-frozen in OCT embedding compound for subsequent immunohistochemistry to determine cells positive for perforin.

Preparation of Effector Cells for ^{51}Chromium Release Assay

Peripheral blood lymphocytes. The PBL were isolated from jugular blood. Briefly, peripheral blood mononuclear cells were purified by density gradient centrifugation on Fico-Lite-1077 at 450 g for 30 min. The cell pellet was resuspended with 4 mL red cell lysis buffer, mixed with a Pasteur pipette for ~ 20 seconds to remove erythrocytes, and diluted with 4 mL double-strength DPBS. Cells were washed twice with 2 mL modified M-199 by centrifugation at 110 g for 5 min. and resuspended with 2 mL modified M-199 [Tissue Culture Medium-199 containing 5% (v/v) horse serum, 200 U/mL penicillin, 0.2 mg/mL streptomycin, 2 mM supplemental glutamine, and 10^{-5} M β-mercaptoethanol].

For the experiment with primary trophoblast, maternal PBL isolated on the day of slaughter were incubated in M-199 in modified T75 flasks at 37°C and 5% (v/v) CO_2 in T75 culture flasks until after the trophoblast cells were cultured with BHV-1. Third-party PBL were collected on either the same day when maternal blood was collected and were processed similarly or were collected and processed as described above on the next day.

Endometrial epithelial cells. Uterine horns were longitudinally opened along the anti-mesometrial border. Luminal epithelium (which contains a mix of epithelial cells and lymphocytes, Majewski et al. 2001) was removed from intercaruncular areas of the endometrium by mechanically scraping the inner surface of the endometrium with a sterile surgical blade. Cell scrapings were collected into a 50 mL sterile culture tube containing 5 mL M-199 supplemented with 2 mM EDTA. Cells were incubated at 37°C for 1 h. Cells in suspension were then triturated vigorously for 5 min to break cell clumps, filtered through a sterile 100 μm cell strainer into 50 mL sterile culture tube and centrifuged at 110 g for 5 min. The cell pellet was resuspended with 5 mL modified M-199 and cell number was determined using a hemacytometer.

Preparation of Target Cells for ^{51}Chromium Release Assay

Primary Trophoblast Cells. After slaughter, the uterus was opened longitudinally along the anti-mesometrial border to expose chorionic membranes. By using sterile forceps, the interplacentomal chorionic membrane layer was separated from the allantois. Small pieces (0.5-2 cm) of chorion collected in this manner were placed into cell culture dishes containing modified M-199 that was also supplemented with 2 mM EDTA, shaken to remove blood, and then placed into new culture dishes containing modified M-199 + 2 mM EDTA. The chorionic tissue was disassociated by scraping with a scalpel blade to

produce a mixture of suspended cells and small pieces of tissue. Cells collected in this manner were incubated at 37°C and 5% (v/v) CO_2 for approximately 1 h. The cells were triturated through a Pasteur pipette and then through an 18 ga needle. The cell suspension was then filtered through a cell strainer (100 μm) to remove tissue clumps. Cells were centrifuged at 110 g for 5 min. The supernatant was discarded and the pellet resuspended with 4 mL of red cell lysis buffer, mixed with a Pasteur pipette for about 20 seconds to remove erythrocytes, and diluted with 4 mL of double strength DPBS. Cells were centrifuged at 110 g for 5 min, resuspended with modified M-199, counted and adjusted to 1x 10^5 cell/mL in modified M-199.

Subcultured Trophoblast Cells. A subcultured trophoblast cell line was produced from freshly isolated primary trophoblast cells from a Day 62 placenta (animal 20) prepared as described above. A 1 mL aliquot of cells was cultured in modified M-199 at 37°C. After 4-5 days, culture medium was replaced with fresh medium and cells were cultured in T75 flasks until confluence at Day 7. Culture medium was then replaced with 10 mL warm trypsin-EDTA. After 3 min at 37°C in 5%(v/v) CO_2, culture flasks were shaken vigorously to detach all cells from the plastic surface. As soon as all cells detached, 10 mL of modified M-199 was added and the cell suspension was centrifuged at 100 g. The supernatant was discarded and cells were resuspended with 4 mL modified M-199. Cells (1x10^6) were split for a total of 2 passages. At the second passage (Day 5), the trophoblast cells consisted of at least two types of cells as determined by microscopic evaluation – fibroblast-like cells and small round cells that were loosely attached and grew in clusters. Flasks containing this mix of trophoblast cells were shaken vigorously without any enzyme treatment and the supernatant containing the loosely-attached cells

was collected and centrifuged at 150 g for 10 min. These cells, termed Saban Tekin ovine trophoblast cell-2 (STOT2) cells, were resuspended in modified M-199 and cultured at 37 °C and 5% (v/v) CO_2. At confluence (4-5 d), they were harvested and split for a new passage. For chromium release assays, STOT2 cells from passage 6 were used. Immunohistochemistry and flow cytometry were performed at passage 7 after subculture.

D17 Cells. Cells were cultured continuously in a modified Eagle's MEM [MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 200 U/mL penicillin and 2 mg/mL streptomycin]. At confluence, D17 cells were trypsinized, mixed with an equal amount of MEM, centrifuged for 5 min at 110 g, resuspended in modified MEM, counted and adjusted to 1 x 10^5 cells/mL in modified MEM.

^{51}Chromium Release Assay

A 1 mL aliquot of 1 x 10^6 target cells (D17 cells, primary trophoblast cells or subcultured trophoblast) was centrifuged at 110 g for 5 min, resuspended with 100 µL modified MEM and 100 µCi $Na^{51}CrO_3$ and incubated for 1 h at 37 °C and 5% (v/v) CO_2. The labeled cells were washed twice with medium and resuspended to 1 x 10^5 cells/mL. Labeled target cells were resuspended to 1 x 10^5 cells/mL and 100 µL of cells pipetted into individual wells of a 96 well flat-bottomed culture plate. Into half of the wells was added 20 µL of the working BHV-1 solution (a 1:10 dilution of the original BHV-1 solution with modified MEM). Plates were incubated at 37°C and 5% (v/v) CO_2 overnight. Cells were then washed twice with 100 µL modified MEM by centrifugation at 80g for 3 min and resuspended with 50 µL modified MEM. An aliquot of 100 µL effector cells (1x10^6, 5x10^5, or 2.5x10^5 PBL or endometrial epithelial cells in modified M-199) were pipetted into wells containing 1x10^4 ^{51}Cr labeled target cells (50 µL) in a

flat-bottom 96-well plate to produce effector: target cell ratios of 100:1 and 50:1. In some experiments, wells also contained either hrIL-2 in DBPS (5 μL to produce a final concentration of 500 U/mL) or an equivalent volume of DPBS as a control. Modified M-199 (45 μL) was added into each well to a final volume of 200 μL. Wells to measure spontaneous release contained 100 μL target cells and 100 μL modified MEM or M-199 only, while wells to measure maximum release contained 100 μL target cells and 100 μL 2% (v/v) Triton X-100.

Plates were centrifuged at 80 g for 3 min to facilitate contact between effector and target cells before incubation at 37°C and 5% (v/v) CO_2 for 20 h. The assay was terminated by centrifugation of plates at 350 g for 15 min at room temperature. A 100 μL aliquot of each supernatant was removed and counted for radioactivity using a gamma counter (Cobra Auto-Gamma, Packard Instrument Inc. Downers Grove, IL). Results of triplicate wells were averaged and expressed as percent lysis using the formula: Percent lysis = (sample DPM – spontaneous DPM) / (maximum DPM – spontaneous DPM) x 100.

Immunohistochemistry

Immunohistochemical staining of STOT2 cells was performed on subcultured trophoblast cells grown in four-well glass chamber slides for 3 d. The supernatant was removed, attached cells washed once with 2 mL staining buffer [10 mM $NaPO_4$, pH 7.4 containing 0.9% (w/v) NaCl (PBS) supplemented with 2% (v/v) normal goat serum], and cells were fixed with 95% (v/v) ethanol. Procedures for immunohistochemistry were carried out according to manufacturer's instructions – all steps were performed at room temperature and cells were washed with staining buffer between all steps. Briefly, cells

were sequentially incubated with blocking buffer [Phosphate buffered saline (PBS) containing 2% (v/v) normal goat serum, 0.3% (v/v) H_2O_2] and tissue conditioner supplied in the kit before incubation with primary antibody for 6 hours. Antibodies used were anti-cytokeratin, SBU-I, anti-vimentin, and control mouse ascites fluid. All antibodies were used at a 1:200 dilution in staining buffer. Slides were then sequentially incubated with secondary antibody (biotinylated anti-mouse immunoglobulin supplied in the kit) for 30 min, streptavidin-alkaline phosphatase reagent (from kit) for 30 min, and 3-amino, 9-ethylcarbazole chromogen reagent for 10 min. Cells were washed under tap water, cover slips mounted, and slides examined for staining using light microscopy (Alphaphot, Nikon, Japan).

Frozen sections of uterine endometrium were prepared for immunohistochemistry by preparing 6 μm sections with a cryostat microtome. Sections were placed onto precleaned glass slides, fixed with 4-5 drops 95% (v/v) ethanol and subjected to immunohistochemical staining. Primary antibodies used were a 1:500 anti-perforin or a 1:500 control mouse ascites fluid as a negative control. Procedures were carried out at room temperature in a humidified chamber as described for STOT2 cells.

Determination of MHC class I Antigen Expression Using Flow Cytometry

STOT2 cells were harvested using trypsin-EDTA, washed twice and resuspended with 2 mL of staining buffer [PBS containing 1% (w/v) BSA and 2% (v/v) fetal calf serum]. Cell number was adjusted to 1x106 cell/mL and $3x10^5$ cells (300 μL) were transferred into 12x75 mm polyethylene culture tubes. Cells were centrifuged and resuspended with 100 μL staining buffer. Primary antibody (5 μL H58A or control mouse ascites fluid) were added into individual tubes and cells incubated on ice for 30 min. Cells were

washed twice, resuspended with 100 μL staining buffer containing 5 μL FITC-labeled, sheep $F(ab)_2$ fragment specific for mouse IgG. After incubation on ice for 30 min in the dark, cells were washed twice and resuspended with 500 μL 1% (w/v) paraformaldehyde. Flow cytometry was performed as soon as possible after staining by using the FACSort flow cytometry device (Becton-Dickinson, Franklin Lakes, NJ). Flow cytometry data were analyzed using CELLQuest software (Becton-Dickinson, Franklin Lakes, NJ).

Statistical Analysis

Data were analyzed by least square analysis of variance using the General Linear Models Procedure of the Statistical Analysis System (SAS, 1998). To determine effects of PBL type (maternal vs third-party) on killing of trophoblast, two analyses were performed. The first included all data. The statistical model included effects of PBL type, trophoblast donor, treatment (control, IL-2 and virus), ratio and all two and three-way interactions. The second analysis was similar except only data from untreated PBL and trophoblast without virus were analyzed. In addition, subsets of data for third-party PBL only and for maternal PBL only were analyzed using effects of trophoblast donor, treatment (control, IL-2 and virus), ratio and all two-way interactions. In initial assays of data from third-party PBL, the effect of pregnancy status of the PBL donor was found to be not significant and this variable was not included in subsequent analyses. For analysis of data for D17 lysis by PBL, the mathematical model included main effects of PBL donor (i.e. ewe), pregnancy status of the PBL donor, IL-2 (+ or -), virus (+ or -), ratio, and all possible interactions. For analysis of data for subcultured trophoblast lysis, the mathematical model included main effects of PBL (ewe), IL-2 (+ or -), virus (+ or -), ratio and all possible interactions. Data on lysis by endometrial epithelial cell

preparations was analyzed using several models. Differences between groups in lytic activity of endometrial epithelial preparations was analyzed using a mathematical model that included effects of source of endometrium (pregnant horn-pregnant uterus, non-pregnant horn-pregnant uterus, and cyclic uterus), ewe nested within source, ratio and all interactions. In addition, another model was performed in which data from both horns of the pregnant ewes were compared to data from cyclic ewes. A smaller subset of data from pregnant ewes only was also analyzed to compare pregnant and non-pregnant uterine horns.

Results

Lysis of Primary Trophoblast Cells by Lymphocytes from Third-Party Donors

Results are shown in Figure 3-1. In the absence of cytokine stimulation, primary trophoblast cells were generally not lysed by PBL from third-party donors. Of the 6 trophoblast cell preparations tested, there was no lysis in 5 cases and only very low lysis ($3.2 \pm 0.93\%$ lysis) in the sixth case. Stated differently, of the 10 third-party PBL preparations (3 pregnant and 7 cyclic ewes), only 1 (a cyclic ewe) showed any lysis towards trophoblast. There was no significant difference in lytic activity between pregnant and cyclic donors.

The trophoblast generally remained resistant to lysis even when preincubated overnight with BHV-1 virus. Treatment of PBL with IL-2 induced large LAK-like activity towards one trophoblast only (control vs IL-2 at 100:1 = 0% vs 31.7%). While third-party PBL did not generally lyse trophoblast, all preparations of PBL used for trophoblast lysis possessed cytotoxic activity against D17 cells, a target for ovine NK cells. Results for a subset of PBL donors in which effects of BHV-1 virus and IL-2 was

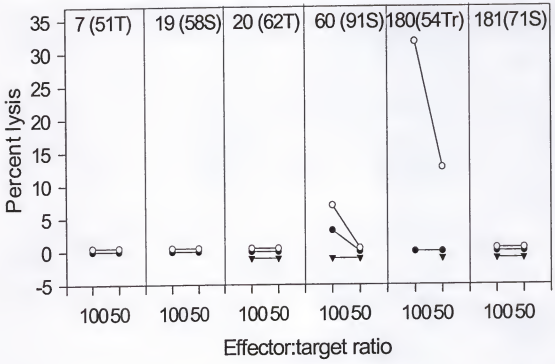


Figure 3-1. Lysis of primary cultures of trophoblast cells by third-party peripheral blood lymphocytes (PBL). Trophoblast cultures prepared from six separate pregnant ewes were subjected to a 20-h ^{51}Cr-release assay using PBL from third-party ewes at effector:target ratios of 50:1 and 100:1. The numbers at the top of each graph are the identification number for the trophoblast donor, the Day of gestation at which trophoblast was collected, and type of pregnancy, singleton (S), twin (T) or triplet (Tr). Each trophoblast preparation was tested in triplicate for lysis using PBL preparations from one (# 60 and #180) or two ewes. Solid circles represent results for untreated PBL, open circles represent results for IL-2-stimulated PBL and solid triangles represent BHV-1 treated trophoblast. Data represent values averaged across all assays. Percent lysis was affected by donor ($P<0.001$), IL-2 ($P<0.001$) and virus x ratio ($P<0.02$).

evaluated are shown in Figure 3-2. Lysis of D17 cells was increased by incubation of D17 cells with BHV-1 virus, especially at effector: target ratios of 100:1 (virus x ratio, $P<0.02$), and by incubation of PBL with IL-2 ($P<0.001$). The magnitude of lysis of D17 cells was similar for PBL from pregnant and cyclic ewes (results not shown).

The proportion of PBL donors that lysed trophoblast tended ($P<0.10$) to be greater for maternal cells (2/4) (Figure 3-3) than for third-party cells (1/10) (Figure 3-1). When comparing results from maternal effectors with results from third-party effectors (Figure 3-1), analysis of variance indicated that lysis was greater for maternal cells (PBL type, $P=0.07$). There were also effects of PBL type x donor ($P<0.0001$), and PBL x treatment ($P<0.04$). When the dataset was restricted to unstimulated cells only (i.e. after excluding IL-2 and virus treatments), there were effects of PBL type ($P<0.0001$), PBL type x donor ($P<0.0001$), and PBL type x ratio ($P<0.02$).

Characteristics of Subcultured Trophoblast Cells

The subcultured trophoblast cell line termed STOT2 was positive for presence of MHC class I as determined by immunohistochemistry using the SBU I monoclonal antibody (Figure 3-4A). Presence of MHC class I was confirmed by flow cytometry – 60% of cells were positive for staining with H58A, another antibody that recognizes sheep MHC class I (Figure 3-5). Immunohistochemistry also revealed expression of both cytokeratin (Figure 3-4B) and vimentin (Figure 3-4C) on STOT2 cells. In contrast to freshly isolated primary trophoblast cells, STOT2 cells were susceptible to lysis by third-party PBL (Figure 3-6). As compared to controls, lysis was enhanced when PBL were treated by IL-2 ($P<0.05$). However, there was no significant increase in lysis when STOT2 cells were infected with BHV-1.

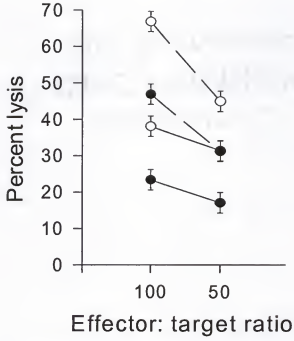


Figure 3-2. Lysis of D17 cells by peripheral blood lymphocytes. Effector cells were the same preparations used for trophoblast lysis studies (Figure 3-1). Data are the least-squares means + SEM of the results from 6 donors. Solid lines represent results for control target cells and dashed lines represent results for BHV-1 infected trophoblast. Solid circles represent results for untreated PBL while open circles represent results for IL-2-stimulated PBL. Lysis was affected by IL-2 ($P<0.001$), ratio ($P<0.001$) and ratio x virus ($P<0.02$).

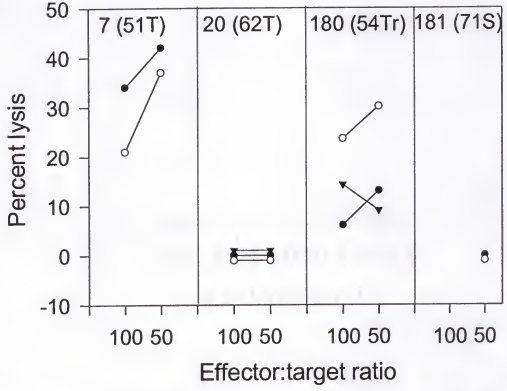
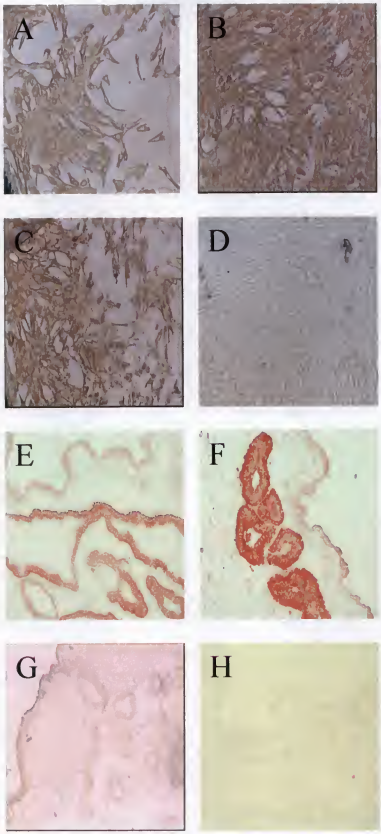


Figure 3-3. Lysis of primary cultures of trophoblast cells by maternal peripheral blood lymphocytes (PBL). Trophoblast cultures prepared from four separate pregnant ewes were subjected to a 20-h 51Cr-release assay using PBL from the same pregnant ewe at effector:target ratios of 50:1 and 100:1. The numbers at the top of each graph are the identification number for the trophoblast donor, the Day of gestation at which trophoblast was collected, and type of pregnancy, singleton (S), twin (T) or triplet (Tr). Solid circles represent results for untreated PBL, open circles represent results for IL-2-stimulated PBL and solid triangles represent BHV-1 treated trophoblast. Data represent values averaged across all assays. Data represent the means of triplicate determinations. Percent lysis was affected by donor ($P<0.01$), donor x treatment ($P=0.05$) and treatment x ratio ($P<0.05$).

Figure 3-4. Immunohistochemical characterization of subcultured placental cells and endometrium. Panels A-D represent immunohistohemical characterization of placental cells using antibodies against MHC class I (SBU-1, panel A), cytokeratin (panel B), vimentin (panel C) and, as a negative control, control mouse ascites fluid (panel D). Panel E-G shows immunohistochemical localization of perforin in endometrium. Represented are tissues from the pregnant (panel E) and non-pregnant (panel F) horns of unilaterally-pregnant ewes (Day 140) and from a cyclic ewe (panel G). The photomicrograph in panel H is of a negative control for perforin immunoreactivity using the same tissue block as panel F and in which control mouse ascites fluid replaced anti-perforin.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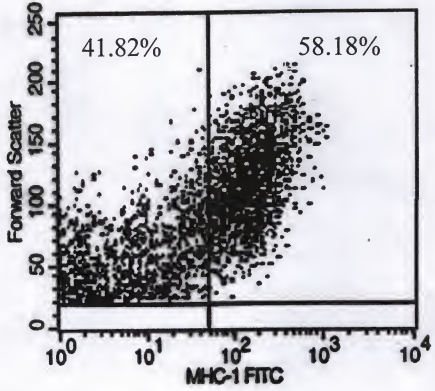

Figure 3-5. Analysis of MHC class I expression on subcultured placental cells by flow cytometry. The antibody used was H58A and the percent positive cells were 57.25% of the total cells.

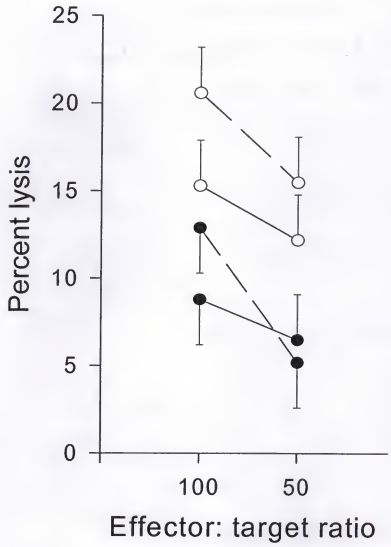

Figure 3-6. Lysis of subcultured placental cells by peripheral blood lymphocytes. Data are the least-squares means $\pm$ SEM of the results from 5 lymphocyte donors. Solid lines represent results for control placental cells and dashed lines represent results for BHV-1 infected placental cells. Solid circles represent results for untreated PBL while open circles represent results for IL-2-stimulated PBL. Lysis was affected by IL-2 ($P<0.05$) but not by virus or the IL-2 by virus interaction.

Presence of NK-like Activity in Endometrial Epithelial Preparations

Preparations of luminal epithelium from the endometrium lysed D17 cells (Figure 3-7). However, there were no significant differences in the amount of killing between preparations derived from the pregnant or non-pregnant uterine horn of unilaterally-pregnant ewes or between these preparations and epithelial cells from cyclic ewes.

To further evaluate the presence of endometrial cells with characteristics of lytic cells, immunohistochemistry was performed using an antibody that recognizes the cytolytic protein perforin. This antibody reacted with luminal and glandular epithelium (Figure 3-4E-F). Immunoreaction product was located throughout the epithelium rather than being limited to specific, isolated cells. Although staining looks more intense in nonpregnant horn, overall there was no difference in intensity of reaction product between pregnant and non-pregnant uterine horns of unilaterally-pregnant ewes or between these tissues and endometrium from cyclic ewes.

Discussion

Results indicate that, like the mouse (Zuckermann and Head 1988) and human (Ferry et al. 1991), the ovine trophoblast is generally resistant to lysis by NK-like cells. As represented I the Figure 3-2, 2/6 trophoblast preparations were lysed by third party PBL and one of these trophoblast preparation (Figure 3-3, #180) was also lysed by third party PBL suggesting that lytic reaction against these trophoblasts is nonspecific. However, the present data provide evidence for the spontaneous generation of maternal cytotoxic lymphocytes against trophoblast during pregnancy (Figure 3-3). These cytotoxic lymphocytes (Figure 3-3, # 7) were likely allospecific since third-party PBL from pregnant females did not lyse trophoblast. The generation of anti-trophoblast cytotoxic lymphocytes during pregnancy differentiates the sheep from the two most-studied

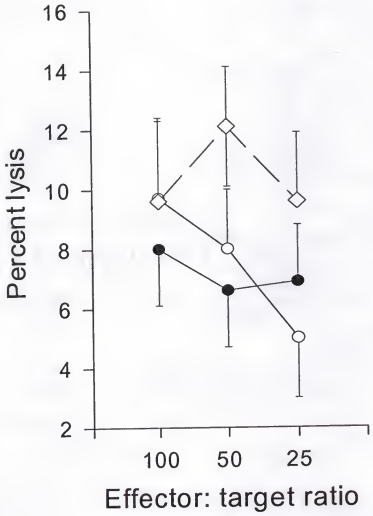

Figure 3-7. Lysis of BHV-1 infected D17 cells by cells in preparations of endometrial luminal epithelium. Data are the least-squares means ± SEM of the results from unilaterally-pregnant and cyclic ewes. Filled circles represent results from the pregnant uterine horn of the unilaterally-pregnant ewe (n=7), open circles represent results from the nonpregnant uterine horn of the pregnant uterus (n=6) and open diamonds represent results from cyclic ewes (n=5). There were no significant differences between pregnant and non-pregnant ewes or between the two uterine horns of the unilaterally-pregnant ewe.

species, the mouse and human, in which it is difficult to generate lymphocyte responses against trophoblast (Blank et al. 1987, Parhar et al 1989, Billington et al. 1993, Yamada et al. 1994). These findings illustrate the potential susceptibility of the trophoblast to specific maternal cytotoxic immune responses and the potential importance for down-regulation of these responses at the maternal-fetal interface for the success of pregnancy in sheep.

The mechanism by which anti-trophoblast cytotoxic lymphocytes are produced during pregnancy is unknown but could include aberrant expression of MHC class I antigens, expression of novel antigenic molecules or lack of expression of molecules that down-regulates the interaction of maternal lymphocytes with trophoblast. The two trophoblast preparations that were lysed by their maternal PBL were from ewes with multiple pregnancies (one twin and one triplicate). It is unknown whether multiple pregnancy might be a contributing factor in development of trophoblast specific lymphocytes. Interestingly, killing of trophoblast by maternal lymphocytes was similar at ratios of 50:1 and 100:1. One explanation for this observation is that activity of maternal cytotoxic lymphocytes was inhibited by suppressor cells in the PBL preparation and that effectiveness of these suppressor cells was lost when PBL were more dilute.

The lack of expression of classical MHC class I antigens on sheep trophoblast cells (Gogolin-Ewens et al. 1989) would be expected to make these cells more susceptible to lysis by NK cells. However, as has been found in mouse (Drake and Head 1989, Zuckermann and Head 1988) and human (King and Kiso 1990, King and Loke 1990, Ferry et al. 1991, Rouas-Freiss et al. 1997, Avril et al. 1999) trophoblast was generally resistant to lysis by NK cells. Moreover, work with B lymphoblastoid cells indicates

susceptibility to bovine NK and LAK cells is independent of expression of MHC class I antigen (Li and Splitter, 1994) and the same may be true for trophoblast. In contrast to the mouse (Drake and Head, 1989) and human (King and Loke 1990, Avril et al. 1999), where IL-2 increased lysis of trophoblast cells by lymphocytes, IL-2 did not generally augment lysis of trophoblast by sheep third-party PBL. Perhaps, longer preincubation times of PBL with IL-2 would have been more effective in inducing LAK cells. However, the incubation time used was sufficient to increase killing of D17 cells by PBL. Thus, it is possible that trophoblast-derived molecules suppressed lymphocyte responses to IL-2 or that LAK-inhibitory molecules exist on the surface of the trophoblast. Consistent with the former hypothesis is the observation that sheep trophoblast secretes molecules that inhibit lymphocyte proliferation (Low et al. 1991). The fact that BHV-1 infection did not increase susceptibility of trophoblast cells to NK cells probably reflects the fact that trophoblast MHC-class I molecules are down-regulated even in the absence of virus. In addition, infected trophoblast cells may not experience increased secretion of IFN-α or IFN-β to modulate NK cell cytotoxic responses (reviewed by Biron et al. 1999). Failure of trophoblast to be killed by NK or LAK cells is not because of the absence of these cytotoxic cells in the sheep because PBL from third-party ewes that did not kill trophoblast did kill D17 cells in a virus- and IL-2-dependent manner.

While presence of HLA-G on trophoblast has been proposed to explain resistance of human trophoblast to NK cell-mediated lysis (Rouas-Freiss et al. 1997, Rouas-Freiss et al. 1999), this explanation will not suffice for all species because since similar molecules have not been reported in non-primates. Even in humans, cells from the JEG-3 trophoblast cell line resisted lysis after acid treatment to remove MHC class I molecules

or after masking HLA-G receptors with monoclonal antibody (Avril et al. 1999). It is thus likely that the trophoblast produces some other NK cell-inhibitory molecule as either a membrane-bound inhibitory receptor or as a soluble mediator of NK cell inhibition.

Unlike primary trophoblast, the subcultured trophoblast cell line that was developed in the present study was susceptible to killing by PBL. One explanation for the increased susceptibility of the STOT2 cells to lysis by NK-like and LAK cells is that the cells underwent some de-differentiation during culture and were no longer capable of synthesis of NK cell-inhibitory molecules. That the cells did experience some de-differentiation is evidenced by the expression of MHC class I which ordinarily is absent on sheep trophoblast (Gogolin-Ewens et al. 1989). Another possibility is that the cell type selected during subculture represents an NK cell-susceptible cell population that is present in low amounts in the mixture of cells making up the primary trophoblast cell preparation. Based on reactivity towards vimentin and cytokeratin, STOT2 cells may be fibroblast-like.

There are few target cells for NK cells in the ruminant and this fact has limited the ability to study NK cell function. Present results indicate that cell lines derived from the trophoblast using procedures as outlined here may be one source for such cells because they were killed by NK-like cells and LAK cells.

In contrast to the results here, trophoblast tissue from peri-implantation sheep conceptuses was lysed by NK cells and prolonged incubation (5 d) with IL-2 generated LAK activity capable of further lysis of trophoblast (Segerson and Gunsett, 1994). While the different culture times (1 vs 5 d) with IL-2 may explain some of these differences, it is probable that the peri-implantation conceptus may be more susceptible to lysis by

cytotoxic cells. In the pig, too, trophoblast from peri-implantation conceptuses is susceptible to lysis by NK cells (Yu et al. 1994). It is likely that trophoblast cells exhibit distinct characteristics at different stages of development that affect their susceptibility to lysis by maternal lymphocytes. For example, trophoblast from preattachment conceptuses might not express inhibitory molecules that suppress cytotoxic cells. The peri-implantation ruminant conceptus may be particularly sensitive to lysis by NK cells because, in addition to being susceptible to lysis by LAK cells (Segerson and Gunsett 1994), the trophoblast at this stage secretes a novel type I interferon called IFN-τ that activates NK cells (Tuo et al. 1993).

Given the potential susceptibility of the trophoblast to specific cytotoxic lymphocytes, and less frequently, LAK cells, the degree to which these cells are present in the uterine endometrium is a pertinent question. There are lymphocytes in the uterine endometrium, almost exclusively located in the epithelium or immediately adjacent to it (Gogolin-Ewens et al. 1989, Meeusen et al. 1993, Majewski et al. 2001). T cells predominate and most of these are $CD8^+$ (Meeusen et al. 1993). The observation in the present study that lytic activity towards D17 cells was observed in preparations of endometrial luminal epithelium indicates that cells capable of nonspecific killing exist in the endometrium. The cell type responsible for this nonspecific killing is unclear. During mid- and late-pregnancy, the number of γδ T cells increases in the luminal epithelium (Lee et al. 1992, Majewski et al. 2001). These cells have been found to contain perforin mRNA (Fox and Meeusen 1999) and as such could possess cytotoxic activity. However, despite the increase in numbers of endometrial γδ T cells during pregnancy, there was no difference in lytic activity between epithelial preparations of pregnant vs non-pregnant ewes. There

may also be NK-like cells in the endometrium, as for other species such as mouse (Croy et al. 1997), human (Ho et al. 1996, King et al. 1996) and pig (Yu et al. 1996), but identification of such cells in the sheep has been made difficult by the lack of antibodies against NK cells.

Another possibility is that a fraction of the epithelial cells themselves can lyse D17 cells. Consistent with this idea was the finding that perforin was expressed throughout the epithelium of the endometrium. Endometrial epithelial cells have been found to play another role analogous to lymphoid cells: in mice, epithelial cells can act as antigen-presenting cells (Sallinen et al. 2000,Wallace et al. 2001). Endometrial epithelial cells can also engage in phagocytosis (Gao et al. 1991) and such an action could potentially lead to lysis of the potential target cells.

Given that the trophoblast is susceptible to specific maternal cytotoxic cells in at least some pregnancies, certain forms of pregnancy loss or reductions in fetal growth may reflect cytotoxic responses mounted by the mother against the placenta. In mice, spontaneous fetal resorption has been correlated with elevated NK cell frequency in some types of matings (reviewed in Clark et al. 1999). Similarly, women with recurrent spontaneous abortion have been reported to have heightened lymphocyte responsiveness to trophoblast antigens (Yamada et al. 1994). It is likely in sheep that one reason that the placenta ordinarily escapes actions of maternal cytotoxic cells is that these cells are inhibited by a progesterone-induced protein secreted by the endometrium called ovine uterine serpin (OvUS). This member of the serpin superfamily can block lymphocyte proliferative responses in response to mitogens and mixed lymphocyte reactions (Segerson et al. 1984, Stephenson et al. 1989, Zhang and Miller 1989) and can block

cytotoxic responses characteristic of NK cells in vitro (Liu and Hansen, 1993, See Chapter 4) and characteristic of NK and LAK cells in vivo (Liu and Hansen, 1993). The trophoblast can also secrete lymphocyte-inhibitory molecules (Low and Hansen, 1988, Low et al. 1991). It is also possible that NK cell or CTL inhibitory molecules are expressed on the surface of the trophoblast. In mice, the trophoblast can inhibit allospecific CTL responses in a manner that is apparently dependent upon cell-cell contact (Zuckermann and Head 1987). It is also possible that generation of anti-trophoblast lymphocytes is limited by mechanisms causing anergy against paternal antigens during pregnancy (Baker et al. 1999, Tafuri et al. 1995) as well as deletion of activated maternal lymphocytes by interactions between lymphocyte Fas and trophoblast Fas ligand (Hunt et al. 1997b, Uckan et al. 1997). Perhaps, the two pregnant ewes that mounted an anti-trophoblast cytotoxic response against their conceptus did so because the trophoblast had some defect in the regulatory system limiting development of maternal responses against the trophoblast. It remains to be seen whether the presence of maternal anti-trophoblast cytotoxic cells compromises pregnancy or whether molecules such as OvUS can block activity of these cells and allow continued trophoblast growth and function.

CHAPTER 4
NATURAL KILLER-LIKE CELLS IN THE SHEEP: FUNCTIONAL CHARACTERIZATION AND REGULATION BY PREGNANCY-ASSOCIATED PROTEINS

Introduction

Natural killer cells participate in innate immunity by exerting nonspecific lysis without prior exposure to antigen against infected or abnormal cells such as tumor cells (Lanier 1998). Natural killer cells can be found in lymphoid tissues such as blood, spleen, bone marrow, and lymph nodes (Trinchieri 1989) and in non-lymphoid tissues such as uterus (Kiso et al. 1992, Ho et al. 1996, Whitelaw and Croy 1996, Croy et al. 1997) and liver (Ljungreen and Karre 1990).

Unlike cytotoxic T cells, NK cells do not require presence of MHC class I molecules on target cells for lysis (Karre 1995). Indeed, MHC class I expression on target cells inhibit NK cell function (Rouas-Freis et al. 1997, Rouas-Freis et al. 1999, Dorling et al. 2000). According to the missing self-hypothesis, NK cells discriminate between cells with self MHC class I versus cells targeted for destruction such as infected cells or tumor cells that have reduced, modified or no MHC class I antigens (Ljungreen and Karre 1990, Karre 1995).

Recognition of a target cell increases intracellular Ca^{++} concentrations in the NK cell, which stimulates release of lytic granules containing perforin and granzymes. Upon release of lytic granules, perforin forms pores into target cell membranes that allows entry of water, salt and granzymes into the cell to cause its lysis (Kagi et al. 1994, Lowin et al. 1995). The importance and function of perforin for NK function have been

demonstrated by treating NK cells with molecules that inhibit perforin processing such as concanomycin-A (CMA), which is an inhibitor of proton pump that maintains acidity in endosomes, ammonium chloride, which increase pH of endosomal compartments (Kataoka et al. 1994, Kataoka et al. 1996), leupeptin, which is a reversible inhibitor of serine and cysteine proteases (Hudig et al. 1984), pepstatin-A, which is an inhibitor of aspartic proteases (Uellner et al. 1997).

To date, there are few reports regarding the presence, regulation or mechanism of action of ovine NK cells. Bovine herpes virus-1 infected D17 cells have been used as an NK cell target in cattle (Li and Splitter 1994). Exposure to BHV-1 increases target sensitivity because it down-regulates expression of MHC class I molecules (Hariharan et al. 1993, Nartaraj et al. 1997) that block NK cell cytotoxicity (Kambayashi et al. 2001, Routes et al. 2001). Sheep PBL can also exhibit NK-like lytic activity against target cells including K562 cells (Tuo et al. 1993) and YAC-1 cells (Liu and Hansen 1993). Such killing differs from human and mouse NK cells in that prolonged incubation with target is required (4 h versus 20 h). In addition, NK cell activity has been reported against ovine preattachment conceptuses (Segerson and Gunsett 1994). The monoclonal antibody, NK5C6 (also called anti Function-Associated Molecule, anti-FAM) produced against nonspecific cytotoxic cells of fish reacts with NK cells from humans and rodents (Evans et al. 1988, Harris et al. 1991, Kapur et al. 1994), and has been reported to react with a subset of lymphocytes in sheep and cattle (Camenisch et al. 1993, Harris et al. 1993).

One potential target for NK cells is the placenta. In many species, including sheep (Gogolin-Ewens et al. 1989), expression of MHC class I and II genes down-regulated on trophoblast, making these cells potential NK targets. Therefore, regulation of NK cell

function during pregnancy might be critical for the protection of the fetal allograft. Natural killer cells have been found in uterine tissues of humans, mice and pigs (Parr et al. 1990, King and Loke 1990, King et al. 1993, Croy et al. 1994). Spontaneous abortions seen in the DBA/2xCBA/J abortion-prone mouse model are associated with infiltration of NK cells into implantation sites (Gendron et al. 1990, Duclos et al. 1994). Additionally, activation of NK cell with polyI•polyC leads to increased abortion in mice (Kinsky et al. 1990, Liu and Hansen 1993). That NK cell-mediated destruction of the conceptus does not usually occur is probably the result of several mechanisms including increased resistance of trophoblast to NK cell-mediated lysis (Zuckermann et al. 1987, Zuckermann et al. 1988, Drake and Head 1989, King and Loke 1990) and production of molecules at the conceptus-maternal interface that down-regulate NK cell function. Several regulatory molecules produced during pregnancy have been implicated in regulation of immune function in sheep. The best studied of these, OvUS, is a member of the serpin family (Ing and Roberts 1989). Among its actions, OvUS can inhibit mixed lymphocyte reactions, mitogen, antigen-induced lymphocyte proliferation of sheep PBL and mouse splenocytes (Segerson et al. 1984, Stephenson et al. 1989, Zhang and Miller 1989, Skopets and Hansen 1993, Skopets et al. 1995). Also it inhibits polyI•polyC induced activation of NK cell and polyI•polyC induced abortion in mice (Liu and Hansen 1993). Recent reports have indicated that OvUS inhibits IL-2-stimulated proliferation of lymphocytes and acts to inhibit lymphocyte proliferation by blocking the upregulation of IL-2 receptor (Peltier et al. 2000c).

In this chapter, it was tested whether activities characteristic of NK-like cells can be identified in ovine PBL and preparations of EEC, whether lytic function of sheep NK

cells can be blocked by inhibitors of perforin processing, and whether lytic function of sheep NK-like cells is regulated by OvUS.

Materials and Methods

Materials

Tissue Culture Medium-199, Eagle's MEM, DPBS, glutamine, penicillin-streptomycin, red cell lysis buffer, β-mercaptoethanol, trypsin-EDTA, Triton X-100, saponin, conconamycin-A, leupeptin, pepstatin-A and guinea pig serum were purchased from Sigma Chemical (St. Louis, MO). Ammonium chloride was from Fisher Scientific (Pittsburgh, PA). Fico-Lite 1077 was from Atlanta Biologicals (Norcross, GA). Horse serum was obtained from Hyclone (Logan, UT). Fetal bovine serum and goat serum were from Intergen (Purchase, NY). $Na^{51}CrO_3$ (specific activity ranged between 258 to 598 mCi/mgCr) was purchased from ICN (Costa Mesa, CA). The D-17 cell line (canine osteocarcinoma) and BHV-1 [TCID for 0.2 mL bovine turbinate cells, 10 d culture, $TCID50 = 10^{6.5}$] were from ATCC (Rockville, MD). Hi-Trap Protein G columns were from Amersham-Pharmacia (Kalamazoo, MI), T75 cell culture flasks were from Sarstedt (Newton, NC), and 96-well flat bottomed Falcon cell culture plates and cell strainer (100 μm) were from Becton Dickinson (Franklin Lakes, NJ). The FlexyMag Separator was obtained from Spherotech, (Schaumburg, IL). Centricon ultrafiltration devices were from Amicon (Beverly, MA).

Antibodies.

Hybridoma cells producing monoclonal antibodies against sheep CD4 (clone 17D), CD8 (clone 7C2), γδ T (clone 86D) and CD45R (clone 73B) were purchased from European Type Cell Culture Collection (Salisbury, UK). Monoclonal antibodies were

used as culture supernatants of hybridoma cell cultures prepared by the Hybridoma Core Laboratory of the University of Florida Interdisciplinary Center for Biotechnology Research. The hybridoma cells producing monoclonal antibodies against human perforin (clone 2d4) were a gift from Dr. Gillian Griffiths (Oxford University, UK). Cell culture supernatant containing anti-perforin was prepared as described as above. Monoclonal antibody NK5C6 (anti-FAM), which is an IgM was HPLC purified and was a gift from Dr. D.L. Evans, (University of Georgia Athens, GA). Goat affinity-purified, phycoerythrin (PE)-labeled $F(ab)_2$ fragment against mouse IgG (whole molecule), sheep affinity-purified FITC labeled $F(ab)_2$ fragment against mouse IgG (whole molecule), mouse ascites fluid (clone NS1), and affinity-purified mouse IgG, guinea pig complement sera were from Sigma Chemical (St. Louis, MO). Affinity-purified mouse IgM was obtained from ICN (Costa Mesa, CA). Goat affinity-purified, PE-labeled antibody against mouse IgM (whole molecule) was purchased from Biomeda (Foster City, CA). Magnetic beads coated with goat anti-mouse IgG (1% w/v, $2x10^8$ particles per mL) were from Spherotech (Schaumburg, IL).

Purification of Monoclonal Antibodies

Monoclonal antibodies were either directly used as culture supernatant or ascites fluid or used after purification from these fluids by affinity chromatography with a Hi-Trap protein G column (volume: 5 mL, capacity: 30 μg). Briefly, the column was prewashed with 2 mL binding buffer (1.5 M Glycine, 1 M NaCl, pH 8.9). Ascites fluid or culture supernatant was diluted 1:10 with binding buffer and 10 mL loaded onto the column at 0.5 mL/min. The column was washed with 25 mL binding buffer and fractions (2 mL) collected. The column was then washed with 20 mL elution buffer (1 M citrate-

phosphate buffer, pH 5.0), fractions were neutralized immediately with 0.5 mL 1 M Tris-HCl, pH 8.8. Immunoglobulin fractions were pooled and concentrated using Centricon ultrafiltration devices. Protein concentration of fractions was determined using the Bradford procedure (Bradford 1976) with mouse IgG as standard.

Purification of Ovine Uterine Serpin

Multiparous ewes that were predominately of Rambouillet breeding (n=12) were prepared to limit pregnancy to one uterine horn as described elsewhere (Bazer et al. 1979). Briefly, one uterine horn was ligated surgically and the ovary ipsilateral to the ligated horn was also removed. After > 30 days of recovery, estrous cycles were synchronized using two injections (i.m.) of 2 mg prostaglandin $F_{2\alpha}$ at 11-day intervals. Ewes were bred at estrus to fertile rams to establish pregnancy. Ewes were slaughtered by captive-bolt stunning at Day 140 of gestation and the pregnant uterus recovered for the collection of uterine fluid.

Ovine uterine serpin was purified from uterine fluid accumulating in the ligated horn as described previously (Moffat et al. 1987). Briefly, uterine fluid was centrifuged at 10000 g for 30 min at 4 °C and stored at –20 °C until purification of OvUS by using cation exchange and gel filtration chromatography. After purification, OvUS was dialyzed against DPBS and concentrated using Centricon ultrafiltration devices. The purity of protein was confirmed by SDS-polacrylamide gel electrophoresis under reducing conditions using 10% (w/v) polyacrylamide gels and detection with Coomassie Blue R-125. The purity of protein was > 90%. Protein concentration was determined using the Bradford (1976) procedure with BSA as standard.

Preparation of Cells for ^{51}Chromium Release Assay

Peripheral blood lymphocytes. The PBL were purified from the buffy coat fraction of heparinized peripheral blood by density gradient centrifugation on Fico-Lite-1077 at 450 g for 30 min. The cell pellet was resuspended with 4 mL red cell lysis buffer, elutriated for ~ 20 seconds to remove erythrocytes, and diluted with 4 mL double-strength DPBS. Cells were washed twice with 2 mL M-199 by centrifugation at 110g for 5 min, resuspended with 10 mL modified M-199 [M-199 containing 5% (v/v) horse serum, 200 U/mL penicillin, 0.2 mg/mL streptomycin, 2 mM supplemental glutamine, and 10-5 M β-mercaptoethanol], and incubated at 37°C and 5% CO_2 for 1 h in T75 cell culture flasks to remove adherent cells. The flasks were shaken vigorously and cells were collected in a 50 mL sterile culture tube. Cells were centrifuged at 110 g for 5 min, resuspended with 5 mL modified M-199 and counted with a hemacytometer.

Endometrial epithelial cells. The uteri were removed from cyclic (n=12) and pregnant (n=1, Day 140) ewes and each uterine horn longitudinally opened along the anti-mesometrial border. The luminal epithelium (which contains a mix of epithelial cells and lymphocytes) was removed from intercaruncular areas of the endometrium by mechanically scraping the inner surface of the endometrium with a sterile surgical blade. Cell scrapings were collected into a 50 mL sterile culture tube containing 5 mL M-199 supplemented with 2 mM EDTA. Cells were incubated at 37°C for 1 h. Cells in suspension were then elutriated vigorously for 5 min to break cell clumps, filtered through a sterile 100 μm cell strainer into a 50 mL sterile culture tube and centrifuged at 110 g for 5 min. The cell pellet was resuspended with 5 mL modified M-199 and cell number was determined using a hemacytometer.

D17 target cells. D17 cells were cultured continuously in a Eagle's MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 200 U/mL penicillin and 2 mg/mL streptomycin. At confluence, D17 cells were trypsinized, mixed with an equal amount of MEM, centrifuged for 5 min at 110 g, resuspended in modified MEM, counted and adjusted to $1x10^6$ cells/mL in modified MEM.

^{51}Chromium Release Assay

A 1 mL aliquot of 1 x 10^6 D17 cells was centrifuged at 110g for 5 min, resuspended with 100 µL modified MEM and 100 mCi $Na^{51}CrO_3$ and incubated at 37°C and 5% CO_2 for 1 h. The labeled cells were washed twice with medium and resuspended to $1x10^5$ cells/mL. Labeled target cells were resuspended to $1x10^5$ cells/mL and 100 µL cells pipetted into individual wells of a 96-well flat-bottomed culture plate. Into half of the wells was added 20 µl working BHV-1 solution (a 1:10 dilution of the original BHV-1 solution with modified MEM). Plates were incubated at 37°C and 5% CO_2 overnight. Cells were then washed twice with 100 µL modified MEM by centrifugation at 80 g for 3 min and resuspended with 50 µL modified MEM.

An aliquot of 100 µL effector cells ($1x10^6$, $5x10^5$, or $2.5x10^5$ PBL or EEC in modified M-199) were pipetted into wells containing $1x10^4$ ^{51}Cr labeled target cells (50 µL) in a flat-bottom 96-well plate to produce effector: target cell ratios of 100:1, 50:1 and 25:1. Wells to measure spontaneous release contained 100 µL target cells and 100 µL modified MEM or M-199 only, while wells to measure maximum release contained 100 µL target cells and 100 µL 2% (v/v) Triton X-100. Plates were then centrifuged at 80 g for 3 min to facilitate contact between effector and target cells before incubation at 37°C and 5% CO_2 for 20 h. The assay was terminated by centrifugation of plates at 350 g for 15 min at

room temperature. A 100 μL aliquot of each supernatant was removed and counted for radioactivity using a gamma counter (Cobra Auto-Gamma, Packard Instrument Inc. Downers Grove, IL). Results of triplicate wells were averaged and expressed as percent lysis using the formula: Percent lysis = (sample DPM – spontaneous DPM) / (maximum DPM – spontaneous DPM) x 100.

Depletion of Lymphocytes by Complement-Mediated Lysis

Freshly isolated PBL ($1x10^7$ cell/250 μL) were incubated with 10 μL ascites fluid containing anti-CD8 or anti-γδ T monoclonal antibodies in 12 x 75 cell culture tubes. Control mouse ascites fluid was used as control. After incubation on ice for 45 min, cells were centrifuged, resuspended and incubated with 200 μL guinea pig complement serum at 37°C for 30 min. Cells were washed once with 2 mL DPBS and resuspended to $1x10^7$ cells/mL with modified M-199. The ^{51}Cr release assay was performed as described above using effector:target ratios of 50:1 25:1. The experiment was repeated three times using PBL from three separate cyclic ewes.

Immunomagnetic Depletion of Lymphocytes

Magnetic beads coated with goat anti-mouse IgG ($2x10^7$ particles) in 250 μL were mixed with 125 μL of either ascites fluid containing anti-CD8 or anti-γδ T or with culture supernatant containing anti-CD4. Beads were incubated with antibody in sterile12 x 75 cell culture tubes at 4 °C for 30 min on a tube rotator at low speed. Controls included incubation of beads with DPBS and control mouse ascites fluid. Beads were then washed twice with 2 mL DPBS for 5 min, resuspended with 250 μL modified M-199 and mixed with 100 μL ($1x10^7$ cell) PBL or EEC on a tube rotator at low speed for 30 min at 4 °C. Magnetic beads were then separated using a FlexiMag Separator. The supernatants

containing unbound cells were transferred to 12 x 75 mm cell culture tubes, centrifuged, resuspended with 1 mL modified M-199 (1x 10^7 cell/mL final) and tested for lysis of D17 target cells at effector:target ratios of 100:1, 50:1 and 25:1. The experiment was performed by using PBL from five cyclic and four pregnant ewes (Day 140) and by using EEC from five pregnant ewes at Day 140 of pregnancy.

Immunoneutralization of NK-Like Activity

For PBL, an aliquot of 100 μL effector cells (containing $1x10^6$ and $5x10^6$ cells in modified M-199) were pipeted into cells containing $1x10^4$ ^{51}Cr labeled BHV-1 infected D17 target cells (50 μL) in a flat-bottomed 96-well cell culture plate to produce effector: target cell ratios of 100:1 and 50:1. Additionally, purified monoclonal anti-CD8 (10 μg/well), anti-γδ T (10 μg/well), anti-FAM (100 μg/well), and cell culture supernatant for anti-CD4 (10 μl/well) were pipetted into designated wells. Isotype controls were mouse IgG (10 μg/well), and mouse IgM (10 μg/well). The experiment was repeated with PBL from five separate cyclic ewes.

For the EEC, 100 μl effector cells containing $1x10^6$ and $5x10^6$ cells in modified M-199 were pipetted into well containing target cells as described above. Effector: target cell ratios were 100:1 and 50:1. The EEC were treated with 10 μL ascites fluid containing monoclonal antibodies against CD8 or γδ T cells, 10 μL cell culture supernatant containing monoclonal antibodies against CD4, 10 μg/well anti-FAM, control mouse ascites fluid (10 μL/well) or mouse IgM (10 μg/well). The ^{51}Cr release assay was carried out as described earlier. The experiment was replicated using EEC from ten cyclic ewes at random stages of the cycle.

Flow Cytometry

To determine expression of FAM, 100 μL aliquot of PBL (5 x 10^5 cells in modified-M-199) was placed into 13x100 mm polyethylene tubes, washed twice with 2 mL of staining buffer [PBS containing 1% (w/v) BSA and 2% (v/v) fetal calf serum], and resuspended with 100 μL staining buffer. Cells were incubated with 5 or 10 μg anti-FAM or mouse IgM (isotype control) on ice for 30 min, washed twice with 2 mL staining buffer, and resuspended with a mixture of 100 μL staining buffer and 10 μL anti-mouse IgG-PE. After incubation for 30 min on ice in the dark, cells were washed twice with 2 mL of staining buffer, resuspended with 500 μL staining buffer. The flow cytometry profiles were obtained on a FACSort flow cytometer (Becton-Dickinson) by using CELLQuest flow cytometry software. The cell populations analyzed were gated on the basis of forward and side (90^o angle) scatter to avoid contamination by dead cells and debris. The analysis was repeated using PBL from three separate cyclic ewes.

For perforin expression, 5x10^6 cells in 100 μL were washed twice with 1 mL staining buffer, and fixed using 2 mL of 1% (w/v) paraformaldehyde in PBS for 15 min at room temperature. Subsequently, 5 x 105 cells in 200 μL were placed into 13x100 mm polyethylene tubes, washed twice with 1 mL staining buffer, and incubated with 1 mL of saponin buffer [PBS containing 0.3% (w/v) saponin and 2% (v/v) goat serum] for 20 min at room temperature. Cells were washed twice with 1 mL saponin buffer and incubated in a total volume of 100 μL saponin buffer containing either 5 μL cell culture supernatant containing monoclonal antibody against human perforin or 5 μL control mouse ascites fluid for 30 min at room temperature. Cells were washed twice with 2 mL saponin buffer and incubated with 10 μL anti-mouse IgG-PE in a total volume of 100 μL saponin buffer

for 30 min at room temperature. Cells were washed twice with 2 mL of saponin buffer and resuspended with 500 μL staining buffer. The flow cytometry profiles were obtained and cell populations analyzed as described earlier. The analysis was repeated using PBL from 4 separate cyclic ewes.

To confirm the effectiveness of magnetic bead depletion, PBL (1×10^6 cells in modified-M199) depleted by using magnetic beads coated with ant-CD8 anti-γδ T and mouse IgG were transferred into 13x100 mm polyethylene tubes, washed twice with 2 mL of staining buffer [PBS containing 1% (w/v) BSA and 2% (v/v) goat calf serum], and resuspended with 100 μL staining buffer. Cells were incubated with 10 μL anti-γδ T, CD8 and mouse IgG (isotype control) on ice for 30 min, washed twice with 2 mL staining buffer, and resuspended with a mixture of 100 μL staining buffer and 10 μL anti-mouse IgG-FITC conjugate. After incubation for 30 min on ice in the dark, cells were washed twice with 2 mL of staining buffer, resuspended with 500 μL staining buffer and flow cytometry analysis files were performed as described earlier.

Immunohistochemistry for FAM and CD45R

Immunohistochemical analysis of luminal endometrium from unilaterally pregnant ewes at Day 140 of pregnancy was performed on 6 μm thick sections of snap-frozen tissue samples. Tissue sections were either fixed with 95% (v/v) ethanol or acetone. Procedures for immunohistochemistry were carried out according to manufacturer's (Biomeda) instructions - all steps were performed at room temperature in a humidified chamber and samples were washed with staining buffer [PBS containing 2% (v/v) goat serum] between all steps. Briefly, samples were sequentially incubated with blocking buffer [PBS containing 2% (v/v) normal goat serum, 0.3% (v/v) H_2O_2] and tissue

conditioner supplied in the kit before incubation with primary antibody overnight. Concentrations of antibody employed were anti-FAM at dilutions in staining buffer ranging between 1:20 to 1:750 and a 1:800 dilution of anti-CD45R in staining buffer. Isotype controls were mouse IgM and control mouse ascites fluid. Slides were then sequentially incubated with second antibody (biotinylated anti-mouse immunoglobulin supplied in the kit) for 30 min, streptavidin-alkaline phosphatase reagent for 30 min, and 3-amino, 9-ethylcarbazole chromogen reagent for 10 min. Slides were washed under tap water, cover slips mounted, and slides examined for staining using light microscopy (Alphaphot, Nikon, Japan). The study was replicated with tissues from 4 unilaterally pregnant ewes. In addition, PBL affixed on slides were used as an additional control tissue.

Effect of Inhibitors on NK-Like Activity of PBL

An aliquot of 100 μL PBL (containing either $5x10^5$ or $2.5x10^5$ cells) were placed into wells containing $1x10^4$ ^{51}Cr labeled BHV-1 infected D17 target cells (50 μL) in a flat-bottomed, 96-well cell culture plate to produce effector: target cell ratios of 50:1 and 25:1. In addition, CMA (100 ng/mL), leupeptin (30 μg/mL), pepstatin-A (10 μg/mL) and amonium chloride (10 mM) were added into designated wells. Control wells included similar volumes of DPBS as used to deliver the inhibitors and final volume was adjusted with modified M-199 to 200 μL. The killing assay was carried out as described earlier. The experiment was performed using PBL from 6 cyclic ewes.

Effect of OvUS on NK-Like Activity of PBL

Aliquots of PBL ($5x10^6$ cell/tube in 100 μL modified M-199) were pipetted into 13x100 mm sterile cell culture tubes. The OvUS was added to designated tubes at a final

concentration of 1 mg/mL. Two separate preparations of OvUS were tested. In addition, control tubes included the same volume of DPBS as used for OvUS and the final volume was adjusted with modified M-199 to 400 μL. The cells were incubated at 37°C and 5% CO_2 for 1 h. Cells were then centrifuged at 110 g for 5 min, supernatant was removed, and the pellet was resuspended with 1 mL fresh modified M-199. Aliquots (100 and 50 μL) of cells were placed with BHV-1 infected D17 cells. The ^{51}Cr release assay was carried out as described earlier. The experiment was performed using PBL from 6 cyclic ewes.

Effect of OvUS on NK-Like Activity of EEC

Aliquots of EEC ($1x10^7$ cell/tube in 250 μL modified M-199) were pipetted into 13x100 mm sterile cell culture tubes and OvUS and OVAL were added to designated tubes at a final concentration of 1 mg/mL. Control tubes were included the same volume of DPBS as used to deliver the proteins and the final volume was adjusted with modified M-199 to 1 mL. The cells were incubated at 37 °C and 5% CO_2 for 1 h, centrifuged at 110 g for 5 min, supernatant removed and the pellet resuspended with 1 mL of fresh modified M-199. The ^{51}Cr release assay was carried out as described earlier. The experiment was performed using EEC from 3 cyclic ewes.

Effect of Pregnancy Status on Activity of NK-Like cells in PBL

Peripheral blood lymphocytes from 9 unilaterally-pregnant ewes at Day 140 of pregnancy and 9 cyclic ewes were tested for lysis of D17 cells using CRA.

Statistical Analysis

Data were analyzed by least square analysis of variance using the General Linear Models Procedure of SAS (SAS, 1998). Analyses included effects of ewe (lymphocyte

donor), treatments, effector:target ratio and all possible interactions. Ewe was considered as a random effect and other main effects were considered fixed. Error terms were determined based on calculation of expected mean squares. For multiple-degree-of-freedom effects, variance was partitioned using orthogonal contrasts to determine individual comparisons. In addition, the pdiff mean separation test of SAS was performed.

Results

Immunoneutralization of NK-Like Activity in Peripheral Blood Lymphocytes and Endometrial Epithelial Cells

As shown in Figure 4.1, lysis of BHV-1 infected D-17 cells by PBL was reduced when incubation was conducted in the presence of anti-FAM ($P<0.001$). In contrast, there was no significant reduction of lysis caused by incubation with anti-CD8, anti-CD4, anti-γδ T or isotype controls (Figure 4-1). In other studies, magnetic bead depletion and complement-mediated lysis using antibodies against CD4, CD8 or γδ T cells did not affect NK-like lysis of BHV-1 infected D-17 cells by PBL (results not shown). The effectiveness of depletion by magnetic bead separation was confirmed by flow cytometry (Percent positive before and after depletion: CD8+, 25.8% + 0.26% vs. 3.65% + 0.20%, γδ T+, 3.06% + 0.26% vs. 0.27% + 0.20%, and mouse IgG, 1.05% + 0.26%). Lysis of BHV-1 infected D-17 cells by EEC was also reduced by coincubation with anti-FAM ($P<0.001$), whereas other antibodies and isotype controls tested did not exert any effect on EEC lytic activity (Figure 4-2).

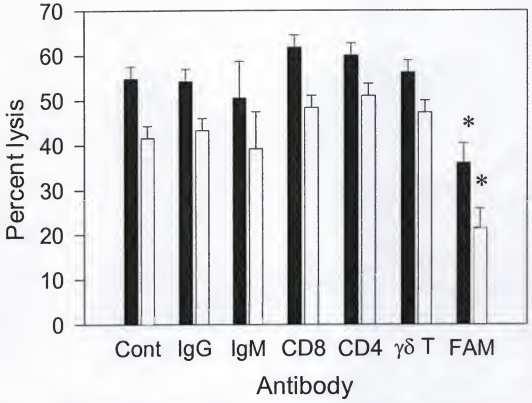

Figure 4.1. Immunoneutralization of NK-like activity in preparations of peripheral blood lymphocytes (PBL). Lysis of bovine herpes virus-1 (BHV-1) infected D17 cells by PBL was conducted in the presence of various antibodies (anti-CD8, anti-CD4, anti-γδ T and anti-FAM), isotype controls (mouse IgG and mouse IgM) or a DPBS control. Lysis was performed at 100:1 (black bars) and 50:1 (open bars). Shown are least squares means ± SEM for results from seven cyclic ewes. Anti-FAM neutralized lysis of BHV-1 infected D17 cells by PBL ($P<0.001$). *Significantly different from control.

Effect of Perforin Processing Inhibitors on Lysis of D17 Cells by PBL

NK-like lysis of BHV-1 infected D17 cells by PBL was significantly reduced by CMA and leupeptin at 50:1 effector: target ratio and by pepstatin-A and amonium chloride at both ratios tested (Figure 4-3).

Perforin Expression in PBL

The percentage of perforin positive cells in PBL (n=4) was 8.83 + 0.68% with flow cytometry (Figure 4-4).

Regulation of NK-Like Lysis of PBL and EEC by OvUS

Both of two separate purification batches of OvUS tested inhibited NK-like lysis of BHV-1 infected D-17 cells by PBL ($P<0.006$) (Figure 4-5A). Lysis of BHV-1 infected D-17 cells by EEC was also inhibited by OvUS ($P<0.05$), but not by the control protein, ovalbumin (1 mg/mL) (Figure 4-5B).

Effect of Pregnancy Status on Lysis of BHV-1 Infected D17 Cells by PBL

Peripheral blood lymphocytes from pregnant (d 140) and nonpregnant ewes were tested for lysis of BHV-1 infected D17 cells. PBL lysed BHV-1 infected D17 cells and lysis increased as ratio of effector:target cells increased ($P< 0.05$). Though there was a tendency for PBL from nonpregnant ewes to cause more lysis, the effect of pregnancy status was not statistically significant (Figure 4-6).

Discussion

The present study confirmed earlier results mentioned in Chapter 3 that sheep PBL, like bovine PBL (Splitter et al. 1988, Wang and Splitter, 1998), can lyse BHV-1 infected D17 cells. As shown in Figure 3-2, lysis of D17 cells was increased by presence of virus and IL-2. Exposure to BHV-1 increases target sensitivity because it down-regulates expression of MHC class I molecules (Hariharan et al. 1993, Nartaraj et al.

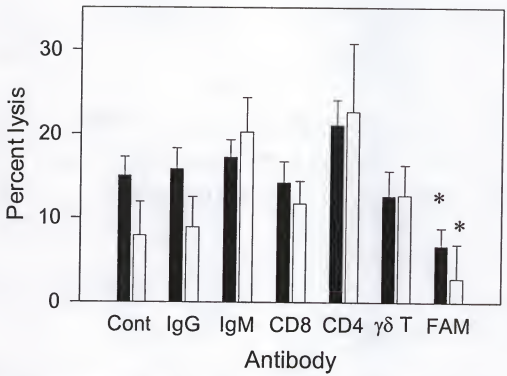

Figure 4-2. Immunoneutralization of NK-like activity in preparations of endometrial epithelial cells (EEC). Lysis of bovine herpes virus-1 (BHV-1) infected D17 cells by EEC was conducted in the presence of various antibodies [anti-CD8, anti-CD4, anti-γδ T anti-FAM), control mouse ascites fluid, mouse IgM or a DPBS control)]. Lysis was performed at 100:1 (black bars) and 50:1 (open bars). Shown are least squares means ± SEM for results from 9 cyclic ewes and 1 pregnant (d 140) ewe. Anti-FAM neutralized lysis of BHV-1 infected D17 cells by EEC ($P<0.001$). *Significantly different from control.

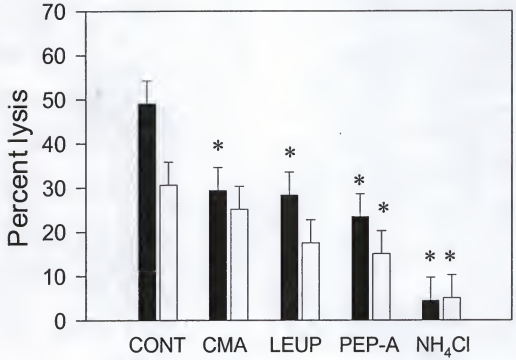

Figure 4-3. The effect of inhibitors of perforin processing on lysis of bovine herpes virus-1 (BHV-1) infected D17 cells by peripheral blood lymphocytes (PBL). The assay was performed with a DPBS control (Cont), concanomycin-A (CMA), leupeptin (LEUP), pepstatin-A (PEP-A) and ammonium chloride. Lysis was at 50:1 (black bars) and 25:1 (open bars). Data are least squares means $\pm$ SEM for results from 6 nonpregnant ewes. As compared to controls, lysis was reduced by all inhibitors at 50:1 and by leupeptin and amonium chloride at 25:1 ($P<0.006$). *Significantly different from control.

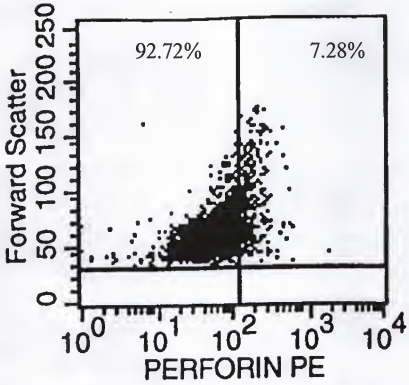

Figure 4-4. The expression of perforin on sheep peripheral blood lymphocytes (PBL). PBL were stained sequentially with mouse anti-human perforin and goat anti-mouse IgG-PE. Shown is a representative result of experiments from five nonpregnant ewes.

1997) that block NK cell cytotoxicity (Kambayashi et al. 2001, Routes et al. 2001). Interleukin-2 activates NK cells and transforms them into LAK cells (Drake and Head 1989). Present studies provide additional evidence that lysis of D17 cells is mediated by an NK-like cell. In particular, lysis was associated with expression of anti-FAM, an NK cell associated molecule, and with perforin activation.

Lysis of BHV-1 infected D17 cells by PBL and EEC was blocked by the presence of antibody against FAM (Figure 4-1 and 4-2), a molecule previously shown to crossreact with NK cell in different species including sheep and cattle (Evans et al. 1988, Harris et al. 1991, Harris et al. 1993, Kapur et al. 1994, Viveiros et al. 1999). Furthermore, results from depletion assays by complement-mediated lysis and magnetic beads indicated that lysis was not caused by cells expressing CD4, CD8 and γδ T cell receptor. Function-associated molecule is a conserved molecule that shares amino acid homology with vimentin, an intercellular cytoskeletal protein found in wide variety of cells. Furthermore, anti-vimentin monoclonal antibodies were able to inhibit NK cell binding and lysis (Karre 1991).

In contrast to reports that anti-FAM labeled a portion of the PBL population by flow cytometry in human, mice, horse, sheep and cattle (Camenisch et al. 1993, Harris et al. 1993, Viveiros and Antczak, 1999), we were unable to identify FAM^+ cells in PBL using flow cytometry or immunohistochemistry or in endometrium by immunohistochemistry. The percent of PBL staining with FAM by flow cytometry (3% + 0.2%) was similar to the proportion of cells nonspecifically stained with the mouse IgM isotype control. Thus, the intensity of reaction of FAM+ cells with antibody is very low or degree of expression is low. Despite use of several different fixatives, there was no detection of FAM+ cells in

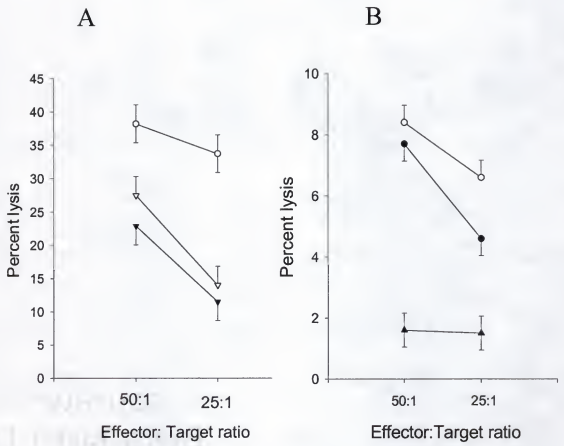

Figure 4-5. Inhibitory activity of ovine uterine serpin (OvUS) on lysis of bovine herpes virus-1 (BHV-1) infected D17 cells by peripheral blood lymphocytes (PBL) (Panel A) and endometrial epithelial cells (EEC) (Panel B). Peripheral blood lymphocytes were pre-incubated with two separate OvUS batches (open and filled triangle) or an equal volume of DPBS (open circle) for 1 h before the ^{51}Cr release assay. Data are least squares means $\pm$ SEM for results by PBL from 6 cyclic ewes at random stages of cycle. Lysis was effected by ratio ($P<0.01$) and treatment ($P< 0.01$). Using orthogonal contrast, OvUS differed from control ($P<0.001$) but there was no difference between the two batches of OvUS. The EEC were cultured with OvUS (filled triangle), OVAL (filled circle) or an equal volume of DPBS (open circle) for 1 h before the ^{51}Cr release assay. Data are least square means $\pm$ SEM for results by EEC from 3 nonpregnant ewes at random stages of cycle. Lysis was effected by ratio ($P<0.01$) and was greater for DPBS control and OVAL as compared to OvUS ($P<0.05$). There was no significant difference between DPBS and OVAL.

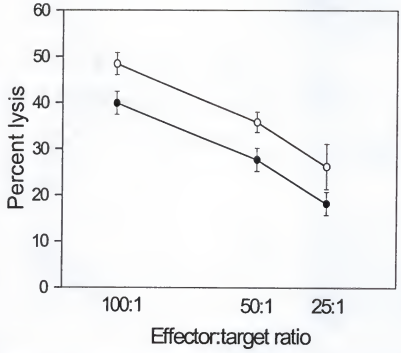

Figure 4-6. Effect of pregnancy status on lysis of bovine herpes virus (BHV-1) infected D17 cells by peripheral blood lymphocytes (PBL). Shown are least squares means of ± SEM of results from 9 pregnant (filled circle) ewes and 9 cyclic ewes (open circle). Lysis was effected by ratio of effector:target cells ($P < 0.05$) but not by pregnancy status.

PBL and endometrium using immunohistochemistry. In contrast, there was positive staining with CD45R as positive control.

Both cytotoxic T cells and NK cells lyse targets through secretion of the pore-forming protein perforin (Lanier and Philips 1992, Kagi et al. 1996). Peripheral blood lymphocytes were treated with known inhibitors of granule acidification and perforin processing to ascertain the role of perforin in lysis of D17 cells. The inhibitors used were conconamycin-A, a proton pump inhibitor, which maintains acidity in endosomes and lytic granules, amonium chloride, which increases the pH of endosomal compartments (Kataoka et al. 1994, Kataoka et al. 1996), leupeptin, a reversible inhibitor of serine and cysteine proteases, and pepstatin-A, an inhibitor of aspartic proteases (Uellner et al. 1997). In previous experiments, CMA and amonium chloride (Savary et al. 1979, Uellner et al. 1997) blocked perforin-mediated lysis by human NK cells. Leupeptin reduced perforin-mediated lysis by inhibiting enzymes involved in processing of inactive perforin to a mature form (Hudig et al. 1984, Uellner et al. 1997). As shown in Figure 4-3, treating sheep PBL with all these molecules reduced lysis of BHV-1 infected D17 cells by PBL. Similarly, Wang and Splitter (1998) demonstrated that lysis of BHV-1 infected D17 cells by PBL in cattle was inhibited by EGTA, a calcium chelator. Therefore, our results support the hypothesis that lytic function of sheep NK-like cells is mediated by proteins, probably perforin, that reside in acidic compartments and require processing for function through cleavage of proteolytic enzymes. The presence of perforin-containing cells in PBL was indicated by flow cytometry where 8.83 + 0.68% of sheep PBL were stained positive for perforin (Figure 4-4).

The NK-like cells found in blood are also likely in the endometrial epithelium. Sheep endometrium contains cells that lyse BHV-1 infected D17 cells and the lysis can be inhibited by anti-FAM (Figure 4-2). Natural killer cells are also found in the endometrium of mice (Head et al. 1994), human (Ho et al. 1996, Whitelaw and Croy 1996) and pigs (Yu et al. 1994). Given that ovine trophoblast cells do not express classical MHC class I and II antigens (Gogolin-Ewens et al. 1989), these cells are susceptible to lysis by NK cells during pregnancy. Indeed, sheep NK-like and LAK cells were cytotoxic towards preattachment ovine conceptuses in early pregnancy (Segerson and Gunsett 1994). Sheep PBL did not kill primary trophoblasts from pregnant ewes at Days 60-91 of pregnancy, but killing could be induced in some cases with IL-2 stimulation (see Chapter 3). Similarly, trophoblast from both mouse (Zuckerman and Head 1988) and human (King and Loke 1990) are resistant to NK cell lysis but susceptible to LAK-mediated killing (Drake and Head 1989, King and Loke 1990). Accordingly, the survival of the conceptus may depend on regulation of cytotoxic effector cells such as NK cells.

One possibility is that NK-like lytic activity of maternal effector cells is regulated through immunoregulatory pregnancy-associated molecules released by the uterus and conceptus. One such molecule is OvUS, which can inhibit a variety of immune functions including inhibition of NK cell cytotoxicity and NK cell-mediated abortion in mice (Liu and Hansen 1993). Consistent with the earlier results, OvUS inhibited lysis of BHV-1 infected D17 cells by both PBL and EEC. This is the first study that demonstrates that OvUS inhibits NK cell activity not only of PBL but also lytic cells from the uterine endometrium (Figure 4-5A/B).

In conclusion, sheep NK-like cells exist in peripheral blood and endometrial epithelium, which can lyse BHV-1 infected D17 cells and which depend on FAM. Their lytic function likely depends on presence and enzymatic activation of perforin or other molecules, which are processed and activated similar to perforin. The endogenous lytic activity of sheep NK-like cells is regulated negatively by OvUS. Regulation of NK-like activity by OvUS is consistent with a role for OvUS in protecting the conceptus from maternal effector cell attack during pregnancy.

CHAPTER 5
DIFFERENCES IN IMMUNOSUPPRESSIVE ACTIVITY AMONG VARIANTS OF OVINE INTERFERON-τ AND REGULATION OF NK-LIKE CYTOTOXICITY

Introduction

Interferon- τ (IFN-τ) is a member of the type I interferon family that also includes IFN-α and β. The closest member of the family is IFN-ω which has 75% amino acid identity with IFN-τ and is 172 amino acids in length (Roberts et al. 1999). Interferon- τ evolved from IFN-ω about 36 million years ago, after the divergence of pecoran ruminants from other artiodactyls (Roberts et al. 1997). Because of their recent evolution, genes for IFN-τ are only found in pecoran ruminants such as sheep, cattle, goats, deer and giraffes (Roberts et al. 1988, Roberts et al. 1997). Tissue expression of IFN-τ is unique among interferons since it is secreted only by the trophectoderm of peri-attachment conceptuses for a limited period in early pregnancy. Peak secretion, which occurs around Days 12–17 in sheep (Leaman and Roberts 1992, Martal et al. 1998) and Days 15 -24 in cattle (Leaman and Roberts 1992, Martal et al. 1998) is coincident with the period when the uterus would ordinarily secrete luteolytic amounts of $PGF_{2\alpha}$. Indeed, its primary role in pregnancy is to inhibit the pulsatile release of $PGF_{2\alpha}$ from the uterus to allow continued survival of the corpus luteum and the maintenance of pregnancy (Hansen et al. 1988, Farin et al. 1990). Interferon-τ also differs from other interferons in that its gene expression is not inducible by virus but rather is expressed constitutively within the trophectoderm (Helmer et al. 1987).

Nonetheless, IFN-τ possesses many biological characteristics of other type I interferons, such as antiviral (Alenko et al. 1997, Roberts et al. 1989) and antiproliferative (Newton et al. 1988, Ealy et al. 1998, Winkelman et al. 1999, reviewed by Demmers et al. 2001) activities, and the ability to induce expression of genes that are normally regulated by other type I IFNs, such as 2', 5' oligoadenylate synthatase (Johnson et al 1999) granulocyte chemotactic protein-2 (Demmers et al. 2001), ubiquitin cross-reactive protein (Johnson et al), Mx protein (Barros et al. 1992), and interferon regulatory factors-1 and -2 (Schmitt et al. 1993). Among the immune cells whose growth is inhibited by IFN-τ are PBL (Newton et al 1989a, Alenko et al. 1997, Bazer et al. 1996, Winkelman et al. 1999) and $WC1^{-}$ $CD8^{+}$ γδ T cells (Tuo et al. 1999). Interferon-τ can stimulate expansion of $WC1^{+}$ γδ T cells (Tuo et al. 1999b) and increase cytotoxic activity of sheep NK cells (Tuo et al. 1993). Regulation of lymphocyte growth and activation may be important for preventing rejection of the allogeneic conceptus, which in the sheep is susceptible to lysis by lymphokine-activated killer (LAK) cells (Ott et al. 1998), or for determining the amount and nature of lymphocyte-derived cytokines capable of regulating conceptus function (Spencer et al. 1998). Immunoregulation of uterine lymphocytes by IFN-τ is indicated by the observation in cattle that during pregnancy, maximal IFN-τ secretion is associated with a decrease in the number of intraepithelial lymphocytes in the endometrium (Tuo et al. 1998).

Multiple genes encode IFN-τ in sheep and cattle and several distinct genes are transcribed during early pregnancy (Martal et al. 1979, Van Der Vielen and King, 1984, Imakawa et al.1987, Roberts et al. 1988, Alexenko et al. 1997, Winkelmann et al. 1999). Recombinant proteins for some forms exist for ovine IFN-τ, and include IFN-τ4, τ6d, τ2c

and τ11 (Nephew et al. 1993, Alexenko et al. 1997, Winkelmann et al. 1999). These proteins differ by no more than 13% in their primary amino acid sequences (Imakawa et al. 1987, Leaman et al. 1992, Nephew et al. 1993 Alexenko et al. 1997). The variants τ4, τ6d and τ2c are apparently equal in relative abundance (Alexenko et al. 1997). Ovine interferon-τ4 is the most potent antiviral IFN-τ (Roberts et al. 1989, Nephew et al. 1993, Alexenko et al. 1997, Winkelman et al. 1999) and is more potent than τ6d and τ11 in inhibiting growth of human Daudi cells (Roberts et al. 1989, Ealy et al. 1998). Moreover, the IFN-τ variants have different potencies in eliciting antiluteolytic actions with τ4 being more effective at extending lifespan of the corpus luteum than τ6d, τ2c, and τ11 (Alexenko et al. 1997, Ealy et al. 1998).

The objectives of this study were to compare IFN-τ variants for potency in ability to inhibit mitogen-induced proliferation of sheep PBL and whether lytic function of sheep NK cell-like cells is regulated by OvIFN-τ and BoIFN-τ.

Materials and Methods

Materials

Adult cycling ewes of predominantly Rambouillet genotype were used as blood donors. TCM-199, Eagle's MEM, DPBS, glutamine, penicillin-streptomycin, PHA, red cell lysis buffer, β-mercaptoethanol, and Triton-X 100 were purchased from Sigma Chemical (St. Louis, MO). Fico-Lite-1077 was from Atlanta Biologicals (Norcross, GA). Horse serum was from Hyclone (Logan, UT). Fetal bovine serum was from Intergen (Purchase, NY). [^{3}H]-methyl thymidine (specific activity: 670 mCi/mmol) was from Amersham Life Sciences (Piscataway, NJ). $Na^{51}CrO_3$ (specific activity ranged between 258 and 598 mCi/mgCr) was purchased from ICN (Costa Mesa, CA). The D-17 cell line

(canine osteocarcinoma) and BHV-1 [TCID for 0.2 mL bovine turbinate cells, 10 d culture, $TCID50 = 10^{6.5}$] were from ATCC (Rockville, MD). T75 cell culture flasks were from Sarstedt (Newton, NC), and 96-well flat-bottomed Falcon cell culture plates were from Becton Dickinson (Franklin Lakes, NJ). Bovine IFN-τ was a gift from Dr. R. Michael Roberts (University of Missouri).

Recombinant Ovine Interferons

Production and purification of recombinant IFN-τ4, τ6d and τ11 were performed as previously described (Ealy et al. 1998). The recombinant ovine IFN-ω protein was generously provided by R.M. Roberts, University of Missouri. The gene was cloned into the pET15b bacterial expression plasmid (Novagen, Madison, WI) and used to transform BL21(DE3) pLysS E. coli (Promega, Madison, WI). Cells were fermented in Luria broth containing ampicillin (50 μg/mL) and chloramphenicol (17 μg/mL) at 37^0C. Recombinant protein expression was induced at the proper cell density ($OD_{600} = 1.0$) with 0.5 mM isopropyl-α-D-thiogalactoside (IPTG) (AlexisCorp. San Diego, CA). After 6 h, cells were collected, lysed and insoluble proteins were isolated and refolded as described previously (De Moraes and Hansen, 1997). Approximately 90% of the resulting protein preparation was estimated to represent ovIFN-ω, as determined visually after SDS-PAGE and Coomassie staining.

Antiviral activity of each recombinant protein was determined as the ability of interferon to inhibit vesicular stomatitis virus-induced cytolysis of MDBK cells (Roberts et al. 1989). One antiviral unit of recombinant ovine interferon was defined as the concentration of interferon that prevented cytolysis by 50%. Using this measure,

antiviral activity was 178.21 pM for τ4, 609.5 pM for τ6d, 330.2 pM for τ11, and 1592 pM for IFN-ϖ.

Preparation of Cells for Lymphocyte Proliferation and ^{51}Chromium Release Assay

Peripheral blood lymphocytes. The PBL were purified from the buffy coat fraction of heparinized peripheral blood by density gradient centrifugation on Fico-Lite-1077 at 450 g for 30 min. The cell pellet was resuspended with 4 mL red cell lysis buffer, triturated for ~ 20 seconds to remove erythrocytes, and diluted with 4 mL double-strength DPBS. Cells were washed twice with 2 mL M-199 by centrifugation at 110 g for 5 min. For lymphocyte proliferation assay, the cells resuspended with 5 mL modified M-199 [TCM-199 containing 5% (v/v) horse serum, 200 U/mL penicillin, 0.2 mg/mL streptomycin, 2 mM extra glutamine and 10^{-5} M β-mercaptoethanol] and counted with a hemacytometer. Cells resuspended at $1x10^6$ cells/mL in a modified TCM-199. For the CRA the cells resuspended with 10 mL modified M-199 and incubated at 37°C and 5% (v/v) CO_2 for 1 h in T75 cell culture flasks to remove adherent cells. The flasks were shaken vigorously and cells were collected in a 50 mL sterile culture tube. Cells were centrifuged at 110 g for 5 min, resuspended with 5 mL modified M-199 and counted with a hemacytometer.

D17 target cells. D17 cells were cultured continuously in a Eagle's MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 200 U/mL penicillin and 2 mg/mL streptomycin. At confluence, D17 cells were trypsinized, mixed with an equal amount of MEM, centrifuged for 5 min at 110 g, resuspended in modified MEM, counted and adjusted to $1x10^6$ cells/mL in modified MEM.

Lymphocyte Proliferation Assay

Briefly, $1x10^5$ PBL in 100 μL total volume were placed in individual wells of 96-well culture plates with various concentrations of IFN-τ variants and 2 μg PHA (dissolved in DPBS, volume 10 μL) in a final volume of 170 μL modified TCM-199. For control wells, an equivalent volume of DPBS was added instead of interferon. After cells were incubated at 37 °C and 5% (v/v) CO_2 for 72 h, [^{3}H] thymidine (0.1 μCi/well) was added in 30 μL modified TCM-199. Cells were incubated overnight at 37 °C and 5% (v/v) CO_2 and then harvested onto glass-fiber filters by using a cell harvester (Brandel, Gaithersburg, MD) and consecutive washes with 0.9% (w/v) NaCl and distilled water. The amount of [^{3}H] thymidine on the filters was measured using a liquid scintillation counter (LKB Wallac Model 1219, Turku, Finland).

In the first experiment, IFN-τ variants were tested at final concentrations (i.e. after addition of [^{3}H] thymidine) of 1, 10 and 20 ng/mL. The experiment was replicated three times with PBL from a different ewe for each replicate. The second experiment was conducted similarly except that concentrations of 20, 60 and 100 ng/mL IFN-τ were tested. For the last experiment, concentrations of τ6d, τ11 and ϖ were tested at antiviral units equivalent to antiviral activity of 1, 10 and 20 ng/mL of τ4. Thus, τ6d [3.42 times less potent than τ4] was tested at 3.42, 34.2 and 68.4 ng/mL, τ11 [1.86 times less potent than τ4] was tested at 1.86, 18.6 and 37.2 ng/mL, and IFN-ϖ [8.94 times less potent than τ4] was tested at 8.94, 89.4, and 178.8 ng/mL. The experiment was replicated three times with PBL from a different ewe for each replicate.

^{51}Chromium Release Assay

A 1 mL aliquot of 1 x 10^6 D17 cells was centrifuged at 110 g for 5 min, resuspended with 100 μL modified MEM and 100 mCi $Na^{51}CrO_3$ and incubated at 37°C and 5%(v/v) CO_2 for 1 h. The labeled cells were washed twice with medium and resuspended to $1x10^5$ cells/mL. Labeled target cells were resuspended to $1x10^5$ cells/mL and 100 μL cells pipetted into individual wells of a 96-well flat-bottomed culture plate. Into half of the wells was added 20 μL working BHV-1 solution (a 1:10 dilution of the original BHV-1 solution with modified MEM). Plates were incubated at 37°C and 5% (v/v) CO_2 overnight. Cells were then washed twice with 100 μL modified MEM by centrifugation at 80 g for 3 min and resuspended with 50 μL modified MEM.

An aliquot of 100 μL effector cells ($1x10^6$, $5x10^5$, or $2.5x10^5$ PBL in modified M-199) were pipetted into wells containing $1x10^4$ ^{51}Cr labeled target cells (50 μL) in a flat-bottomed 96-well plate to produce effector: target cell ratios of 100:1, 50:1 and 25:1. Wells to measure spontaneous release contained 100 μL target cells and 100 μL modified MEM only, while wells to measure maximum release contained 100 μL target cells and 100 μL 2% (v/v) Triton X-100. Plates were then centrifuged at 80 g for 3 min to facilitate contact between effector and target cells before incubation at 37°C and 5% (v/v) CO_2 for 20 h. The assay was terminated by centrifugation of plates at 350 g for 15 min at room temperature. A 100 μL aliquot of each supernatant was removed and counted for radioactivity using a gamma counter (Cobra Auto-Gamma, Packard Instrument Inc. Downers Grove, IL). Results of triplicate wells were averaged and expressed as percent lysis using the formula: Percent lysis = (sample DPM – spontaneous DPM) / (maximum DPM – spontaneous DPM) x 100.

Effect of IFN-τ on NK cell-like Lysis of PBL

Aliquots of PBL ($5x10^6$ cell/tube in 100 μL modified M-199) were placed in 13x100 mm sterile cell culture tubes and OvIFN-τ and BoIFN-τ added to designated tubes at a final concentration of 100 ng/mL. Control tubes included the same volume of DPBS as used to deliver the cytokines and the final volume was adjusted with modified M-199 to 500 μL. The cells were incubated at 37 °C and 5% (v/v) CO_2 for 1 h, centrifuged at 110 g for 5 min, supernatant removed and the pellet resuspended with 1 mL of modified M-199. Aliquots (100 and 50 μL) of cells were placed onto BHV-1 infected D17 cells. The ^{51}Cr release assay was carried out as described earlier and performed using PBL from 6 separate cyclic ewes.

Statistical Analysis

Data were analyzed by least squares analysis of variance using the General Linear Models procedure of the Statistical Analysis System (SAS, 1998). Ewe was considered a random effect and other main effects were considered as fixed. Differences in proliferation between control lymphocytes and individual concentrations of each interferon were determined by the pdiff mean separation test of SAS. To determine differences in potency of individual interferons, the data set was restricted to exclude data for control lymphocytes and orthogonal contrasts were used to determine differences between interferons. The contrasts were IFN-τ4 vs. others, τ6d + τ11 vs. ϖ and τ6d vs. τ11.

Results

Inhibition of Mitogen-Induced Proliferation of PBL by IFN-τ Variants

When IFN-τ variants were tested at 1, 10 and 20 ng/mL, all concentrations of each

IFN-τ variant inhibited [^{3}H] thymidine uptake by PHA-stimulated PBL ($P<0.05$). However, IFN-τ4 was more inhibitory than either IFN-τ6d, IFN-τ11 or an IFN-ϖ control (treatment x concentration, $P<0.03$). For example, [^{3}H] thymidine incorporation averaged 23008 dpm for controls, 13000 dpm for 20 ng/mL τ4, 15155 for 20 ng/mL τ6d, 16801 dpm for 20 ng/mL τ11 and 18583 for 20 ng/mL IFN-ϖ (SEM= ±898) (Figure 5-1, left panel).

A similar result was obtained when IFN-τ variants were tested at concentrations of 20, 60 and 100 ng/mL (Figure 5-1, right panel). All concentrations of each interferon inhibited [^{3}H] thymidine uptake ($P< 0.025$), IFN-τ4 was more inhibitory than the other interferons ($P< 0.001$) and IFN-ϖ was the least inhibitory (τ4 vs. τ6d + τ11, $P< 0.001$).

The inhibition of proliferation was dose-dependent. However, inhibition plateaued at 60 ng/mL and addition of 100 ng/mL did not cause further inhibition Moreover, at the highest concentrations tested, inhibition was only ~50 % for τ4, 36 % for τ6d, 31.5 % for τ11 and 15 % for ϖ.

Differences in activity of IFN-τ variants were also apparent when concentrations of each interferon was adjusted for its antiviral activity. Again, all concentrations of each interferon inhibited [^{3}H] thymidine uptake ($P< 0.001$) (Figure 5-2). However, even though concentrations of interferons were adjusted for differences in antiviral potency, IFN-τ4 was still more inhibitory than other interferons ($P<0.01$) and ϖ was the least inhibitory ($P<0.02$).

Regulation of NK-Like Lysis of PBL by IFN-τ

As compared to DPBS controls, ovine and bovine IFN-τ (100 ng/mL) significantly increased lytic ability of PBL against BHV-1 infected D-17 cells ($P<0.05$) (Figure 5-3).

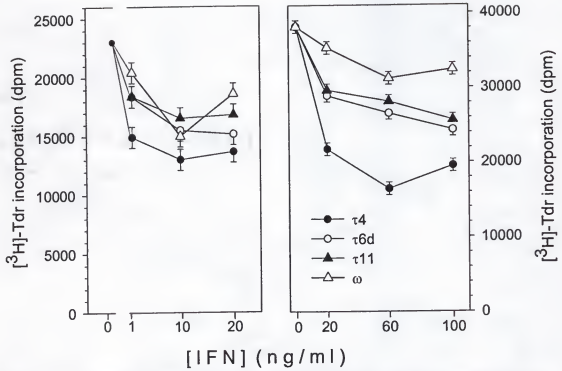

Figure 5.1. The effect of ovine interferon (ovIFN) -τ variants on phytohemagglutinin-induced proliferation of peripheral blood lymphocytes at equal concentrations. Lymphocytes were incubated for 72 h with ovIFN-τ variants and ovIFN-ϖ, pulsed with [^{3}H] thymidine overnight and harvested. Results are the least-squares means ± SEM of results from three ewes.

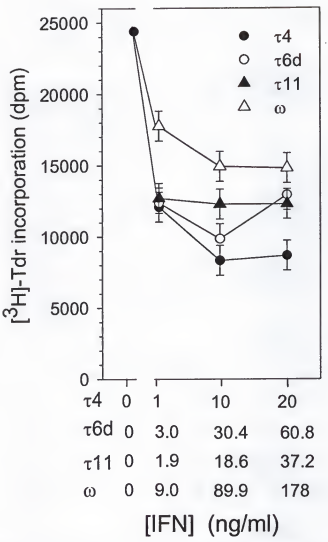

Figure 5.2. The effect of OvIFN variants on phytohemagglutinin-induced proliferation of peripheral blood lymphocytes at adjusted equal antiviral concentrations. Lymphocytes were incubated for 72 h with ovIFN-τ variants and ovIFN-ϖ, pulsed with [^{3}H] thymidine overnight and harvested. Results are the least-squares means ± SEM of results from three ewes.

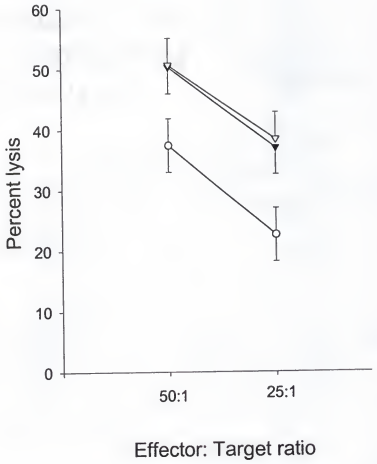

Figure 5-3. Effect of Interferon-τ (IFN-τ) on lysis of bovine herpes virus-1 infected D17 cells by peripheral blood lymphocytes (PBL). Before the ^{51}Cr release assay, PBL were pre-incubated with ovine (filled triangle) and bovine IFN-τ (open triangle) or an equivalent volume of DPBS (open circle) for 1 h. Shown are least squares means ± SEM for using 6 separate cyclic ewes. The lytic activity was affected by ratio ($P=0.05$) and treatment ($P<0.01$). Using orthogonal contrasts, the two interferons were different from controls ($P<0.01$) but there was no difference between bovine and ovine IFN-τ.

Discussion

It has been previously reported that OvIFN-τ variants showed differences in antiviral and antiluteolytic activity (Roberts et al. 1997, Alenko et al. 1997, Ealy et al. 1998) with τ4 being the most active variant. Parallel to these reports, present results indicate that IFN-τ variants τ4, τ6d, τ11 and IFN-ϖ vary in effectiveness in inhibiting PHA-induced proliferation of lymphocytes with IFN-τ4 being the most potent inhibitor among the variants tested. Fillion et al. (1999) showed that five variants of IFN-τ purified from conditioned medium of cultured sheep conceptuses were immunosuppressive towards PHA-induced lymphocyte proliferation. However, differences among these variants in inhibiting proliferation was not evaluated.

The well-studied observation that IFN-τ, like other type I interferons, can inhibit lymphocyte proliferation (Fillion et al. 1999, Newton et al. 1989a, Skopets et al. 1992) has been interpreted to indicate that one function of IFN-τ is to block maternal lymphocyte responses directed against the conceptus. Indeed numbers of intraepithelial lymphocytes in the luminal epithelium decline from Day 20 until Day 27 of pregnancy, when IFN-τ secretion is maximal (Tuo et al. 1999a). However, this interpretation might be over-simplified since type I interferons have been shown to augment skin allograft rejection (Benizri et al. 1998, Tovey et al. 1996) and activate NK cells (Tuo et al. 1993), actions which could compromise immunotolerance towards the conceptus. The nature of the effect of IFN-τ probably depends upon the particular lymphocytes in the uterine epithelium. For example, IFN-τ stimulates $WC1^+$ $CD8^+$ γδ T cells and suppresses $WC1^-$ $CD8^+$ γδ T cells (Tuo et al. 1999b).

Lytic activity of NK cells can be modulated through type I interferons such as IFN-α, β (reviewed by Biron 1999) and -τ (Tuo et al. 1993). Consistent with these reports is the present finding that both OvIFN-τ4 and BoIFN-τ enhanced NK-like lysis of BHV-1 infected D17 cells by sheep PBL (Figure 5-3). Perhaps, secretion of IFN-τ by preimplantation conceptuses is one reason the preattachment conceptus is susceptible to NK and LAK cells (Segerson and Gunsett 1994). In contrast, lack of OvIFN-τ secretion in trophoblast prepared from mid-gestation conceptuses may explain why this tissue is generally not killed by sheep NK-like cells (see Chapter 3). While the pre-attachment conceptus is at risk to lysis from NK cells, it may be protected from such cells by other immunosuppressive molecules. One such molecule is a lactosaminoglycan-containing protein produced by the peri-attachment sheep conceptus that can block lymphocyte proliferation (Newton et al. 1989a). Indeed, some activation of NK cells may be beneficial for pregnancy. Knockout mice without NK cells had reduced litter size associated with perturbations of the vascular bed in the placenta (Guimond et al. 1997). Additionally, it has been demonstrated in mice that interferon-γ released from NK cells was beneficial for pregnancy (Ashkar and Croy 1999, Ashkar and Croy 2001).

In conclusion, sheep trophectoderm secretes variants of IFN-τ that differ in their ability to regulate maternal lymphocyte function. Thus, the nature of the effect of the trophectoderm on endometrial lymphocytes may depend upon the type and the relative amounts of each IFN-τ variant produced during pregnancy. The differences in the ability of IFN-τ variants to regulate immune cells may explain why multiple variants of IFN-τ are expressed during early pregnancy. Perhaps distinct variants are responsible for conferring different physiological functions. For example, some variants may be better

able to regulate uterine prostaglandin secretion while others may have evolved to serve other important functions, such as regulating the function of immune cell populations within the uterus during peri-implantation. In addition, the endogenous lytic activity of sheep NK-like cells is regulated positively by OvIFN-τ and BoIFN-τ. Activation of NK cells may be a consequence of IFN-τ secretion by the peri-attacment conceptus may be beneficial for conceptus.

CHAPTER 6
GENERAL DISCUSSION

The goal of this dissertation was to understand the role of NK cells in pregnancy immunology in the sheep. As stated in the Introduction, specific hypotheses were to test 1) whether lymphocytes from peripheral blood and endometrium possess NK-like cytotoxic activity, 2) NK-like cells and T cells in peripheral blood can lyse trophoblast, 3) NK-like function of PBL is mediated by lytic molecules, such as perforin and 4) NK-like cells in PBL and endometrium are regulated by OvUS and IFN-τ. Results of the research described in this thesis suggest that NK cells or cells with properties similar to NK cells exist in sheep and that they are potentially regulated by products of the trophoblast and uterus in a way that supports maintenance of pregnancy.

Several lines of evidence suggest that PBL and the endometrium contain an NK-like cell. First, PBL lysed an NK cell target, the D17 cell (Figure 3-2). In addition, infection of D17 cells with BHV-1 increased killing (Figure 3-2) and treatment of PBL with IL-2 enhanced lytic activity against D17 cells (Figure 3-2). Endometrial epithelial cell preparations were also capable of lysing BHV-1 infected D17 cells (Figure 3-7). Furthermore, as shown in Figure 4-2, cytotoxic activity of PBL and endometrial cell preparations against BHV-1 infected D17 cells was significantly reduced by immunoneutralization with anti-FAM, an antibody that recognizes a vimentin-like antigen expressed on NK cells in other species including human and mouse (Evans et al. 1988, Harris et al. 1991, Kapur et al. 1994). Finally, killing of D17 cells by PBL was reduced by molecules that inhibit perforin processing (Figure 4-3).

Given the probability that NK cells or cells similar to NK cells exist in the sheep, the fact that MHC class I molecules are absent on ovine trophoblast (Gogolin-Ewens et al. 1989) makes these cells potentially susceptible to killing by NK cells. However, as has been described earlier for human and mouse trophoblast (Zuckerman and Head 1988, Drake et al. 1989, Ferry et al. 1991, Avril et al. 1999), primary sheep trophoblast was generally resistant to NK-like lysis of PBL even when NK cells were activated by IL-2 (Figure 3-1).

Why then doesn't killing of the trophoblast take place? Two potential mechanisms suggested from research in this thesis are the differentiation of the trophoblast and the secretion of OvUS by the endometrium. The fact that subcultured trophoblast becomes susceptible to killing by NK-like cells (Figure 3-6) points out that the trophoblast must possesses some mechanism for inhibition of NK cell responses that is lost upon de-differentiation. In the human, presence of HLA-G on trophoblast has been proposed to explain resistance of trophoblast to NK cell-mediated lysis (Rouas-Freiss et al. 1997). This explanation will not suffice for all species since similar molecules have not been reported in non-primates. Even in humans, cells from the JEG-3 trophoblast cell line resisted lysis after acid treatment to remove MHC class I molecules or after masking HLA-G receptors with monoclonal antibody (Avril et al. 1999). Thus, it is possible that other NK or LAK cell inhibitory molecules exist on the cell membrane of trophoblast. Several NKIM have been identified on trophoblast cells, including HLA-C, -G, and –E (reviewed by Fernandez et al 1999). It will be important to determine whether these molecules are expressed on trophoblast. It is also important that whether NK inhibitory receptors are expressed on sheep NK cells.

Secretory molecules from endometrium and trophoblast may also be involved in immunoregulation of NK cells. Among these, OvUS is the most studied immunoregulatory molecule produced by endometrium in sheep. Ovine uterine serpin can inhibit the mixed lymphocyte reaction and mitogen and antigen-induced lymphocyte proliferation (Segerson et al. 1984, Stephenson et al. 1989, Zhang and Miller 1989, Skopets and Hansen 1993, Skopets et al. 1995). Present results indicate that OvUS inhibited lysis of D17 cells by PBL (Figure 4-5A) and EEC (Figure 4-5B). These results are consistent with earlier reports that OvUS inhibited sheep NK cell lysis of K562 cells and inhibited polyI•polyC-activation of mouse splenocytes and abortion in vivo (Liu and Hansen 1993). Trophoblast-derived soluble immunoregulatory molecules may also regulate NK cell function. In fact, the sheep trophoblast produces molecules that inhibit lymphocyte proliferation (Newton et al. 1988, Newton et al. 1989a, Low et al. 1991). Dosogne et al. (2000) has recently shown that bovine PAG inhibited proliferation of bone marrow progenitor cells, suggesting that PAG may also be involved in immunoregulation during pregnancy.

One dichotomy of the present results was that the peri-implantation conceptus secretes a molecule, IFN-τ, that actually enhances NK cell activity (Figure 5.3). Such an observation raises two question - are there other molecules to prevent IFN-τ induced activation of NK cells or do the activated NK cells play some beneficial role at this stage of pregnancy. It is not known whether other immunosuppressive molecules inhibit NK function but endometrium produces immunosuppressant PGE_2 during early pregnancy (Challis et al. 1982) and the conceptus produces immunosuppressive lactosaminoglycan at this stage (Newton et al. 1989a). There is abundant evidence, however, for some

beneficial effects of products of activated NK cells on the outcome of pregnancy. Indeed, GM-CSF, which is produced by activated NK cells, promotes placental growth in humans and mice (Drake and Head 1994, Jokhi et al. 1994). The litter sizes of GM-CSF$^{-/-}$ x GM-CSF$^{-/-}$ breeding pairs were 25% smaller at weaning than control pairs, due to fetal death late in gestation and early in postnatal life (Robertson et al. 1999). In addition, LIF is also produced by NK cells in mouse (Chaudhury and Knap 2000) and is required for implantation. In fact, LIF $^{-/-}$ mice are fertile but the blastocyst cannot attach to uterine epithelium although LIF $^{-/-}$ blastocysts can implant in a normal pseudopregnant mouse (Croy et al. 1991, Stewart et al. 1992). Similarly, LIF has been found in sheep endometrium during early pregnancy with expression highest on days 16-20 (Vogiagis et al. 1997).

Interferon-γ is another cytokine proposed to regulate implantation, vascular remodeling, and decidual integrity in mice (Ashkar and Croy 1999, Ashkar et al. 2000). Indeed, TgE26 mice that lack NK cells in the uterus displayed reproductive compromise characterized with decidual disintegrity, lack of vascular remodeling and development, and reduced embryo size as compared to normal mice (Guimond et al. 1997, Ashkar et al. 2000, Greenwood et al. 2000). Another strain of NK-deficient mice, RAG-2$^{-/-}$/ $\gamma_c^{-/-}$ mice (NK-, T-, B-), had similar placental and decidual defects as did TgE26 mice (Ashkar and Croy et al. 2001). Reconstitution of RAG-2$^{-/-}$/ $\gamma_c^{-/-}$ mice with bone marrow from SCID mice (NK+, T-, B-) resulted in normal decidual development and vascular remodeling (Ashkar end Croy 2001). In both these mouse models, there was lack of expression of IFN-γ in implantation sites (Wang et al. 1994, Ashkar and Croy 2000, Ashkar and Croy 2001). In addition, IFNγ$^{-/-}$, IFN-γRα$^{-/-}$ and Stat-1$^{-/-}$ mice showed similar implantation site

defects as that observed in uterine NK deficient mice strains (Ashkar and Croy 1999). Thus, it is possible that sheep NK cells are stimulated in early pregnancy to support placental growth, attachment, and vascular development during pregnancy.

Based on the literature and the current results, an outline of the immunological situation at the maternal-conceptus interface at certain key stages of pregnancy can be sketched (Figure 6.1). Three such periods will be described. The first is the peri-implantation period, around day 15 of pregnancy, when the conceptus is involved in regulation of corpus luteum lifespan through secretion of IFN-τ. The second period is mid pregnancy, around Day 75 of gestation, when the placenta has been established and the uterus is under domination by progesterone and secreting copious amounts of OvUS. The third period is at term, at Day 147 of gestation, when the local concentration of both progesterone and OvUS is declining, the endometrial epithelium is rich in apparently-activated γδ T cells, and parturition is impending. The proposed events for different stages of pregnancy are summarized in Figure 6-1, -2, and -3.

At Day 15, the conceptus is highly elongated but not attached to the uterine endometrium (Figure 6-1). The antigenicity of the trophoblast at this time is not known but, by Day 19 of pregnancy, MHC class I molecules are not found on trophoblast (Gogolin-Ewens et al. 1989). Like human trophoblast (Drake et al. 1989), the peri-implantation sheep trophoblast is susceptible to lysis by LAK cells (Segerson and Gunsett 1996), suggesting that the trophoblast has not acquired any specialized function such as expression of NK inhibitory receptors to prevent NK cell killing. Thus, transformation of NK cells into LAK cells may result in killing of trophoblast. The key immunological event at this time is the secretion of IFN-τ by trophoblast, which although it possesses

antiluteolytic activity and inhibits endometrial prostaglandin secretion (Demmers et al. 2001), also has potent immunoregulatory actions (Newton et al. 1989a, revieved by Demmers et al. 2001). Paradoxically, one action of IFN-τ is to activate NK cells (Figure 5-3). As alluded to earlier, this finding poses two questions: what is the function of IFN-τ-activated NK cells and why isn't trophoblast destroyed by these NK cells? One possibility is that activated NK cells produce cytokines that stimulate growth of the trophoblast, promote placentation and regulate vascular remodeling of endometrium. As mentioned previously, the effect of NK cells could be mediated by secretion of GM-CSF (Drake and Head 1994, Jokhi et al. 1994), LIF that is produced by NK cells (Bulmer et al. 1991), and IFN-γ (Ashkar and Croy 1999, Ashkar et al. 2000). Since the Day 15 conceptus is susceptible to NK cell lysis and secretes a molecule that activates NK cells, some other immunoregulatory molecule may down-regulate or limit NK cell lytic function. At this period of gestation, the trophoblast secretes a high molecular weight glycoprotein (HMWGP) that can inhibit T blastogenesis (Newton et al. 1989a, Newton et al. 1988). Perhaps this molecule or another molecule inhibits or limits NK cell function. It is unlikely that progesterone is important for NK cell biology at Day 15. The concentration of progesterone in the blood is insufficient to directly inhibit lymphocyte function (Low and Hansen 1988) and secretion of OvUS at this stage of pregnancy is low (Ing et al. 1989, Stewart et al. 2000).

By Day 75, the immunological status in the uterus has changed (Figure 6-2). The trophoblast remains susceptible to lysis by NK cells because of lack of MHC class I molecules (Gogolin-Ewens et al. 1989). In some cases, maternal cytotoxic T cells develop against the conceptus (Figure 3-3), suggesting that other antigens exist on

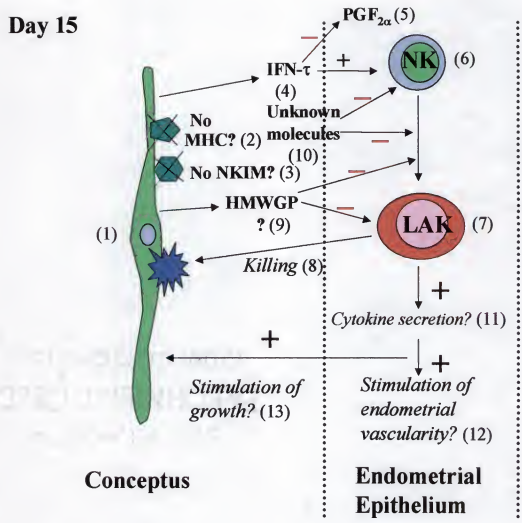

Figure 6-1. Proposed immunological status of the uterus at Day 15 of pregnancy in the sheep. The pre-attachment conceptus (1) does not express MHC class I molecules (2) or NK inhibitory molecules (3). As a result, the trophoblast is a potential target for NK cells. The predominant secretion of the trophoblast is IFN-τ (4) which inhibits endometrial $PGF_{2\alpha}$ secretions (5) to block luteolysis. This cytokine can also stimulate activation of NK cells (6) to LAK cells (7) that can otherwise kill trophoblast (8). Secretion of molecules such as HMWGP (9) and other unknown molecules (10) from trophoblast and endometrium may also be involved in inhibition of NK cells or LAK cells. In addition, NK and LAK cells may secrete cytokines (11) and thereby stimulate endometrial vascularity (12) and placental growth (13).

trophoblast that can lead to immune response. However, there are both endometrial and trophoblast-derived mechanisms to prevent killing of trophoblast. Of key importance is induction of OvUS secretion from endometrial glands by prolonged progesterone exposure. By Day 100 of gestation, OvUS is the major product of the endometrium (Moffat et al. 1987, Stephenson et al. 1989). This protein can regulate a variety of immune responses such as inhibition of mixed lymphocyte reactions (Segerson et al. 1984), mitogen-induced lymphocyte proliferation (Skopets and Hansen 1993, Skopets et al. 1995), and polyI•polyC induced activation of NK in mice (Liu and Hansen 1993). As shown in Figure 4-5, OvUS also inhibits NK-like cytotoxicity in PBL and endometrial epithelium against BHV-1–infected D17 cells. Such a result, which is consistent with earlier findings that OvUS blocks NK cell-mediated abortion in mice (Liu and Hansen 1993), indicates that OvUS regulates NK cell function for the maintenance of pregnancy.

While progesterone can thus regulate uterine immune responses through induction of OvUS, it is unclear whether direct effects of progesterone on lymphocytes occur. After Day 50, ovariectomy does not cause abortion (Casida and Warwick 1945, Beal et al. 1986) so placental progesterone synthesis is sufficient to maintain pregnancy. Even though progesterone does not inhibit lymphocyte proliferation until concentrations reach 10^{-5} to 10^{-6} M (Low and Hansen 1988, Monterreso and Hansen 1993), local concentrations at the maternal-fetal interface at Day 75 are probably high enough to inhibit lymphocyte function. In humans, progesterone stimulates secretion of a protein called PIBF from lymphocytes that in turn can inhibit NK cell function (Szekeres-Bartho et al. 1989, Szekeres-Bartho and Wegmann 1996).

Another product of the placenta that may down-regulate lymphocyte function is PGE_2, which is produced by endometrium and placenta throughout most of pregnancy (Challis et al. 1982) and can inhibit mixed lymphocyte reaction and mitogen-induced proliferation of lymphocytes (Low and Hansen 1988). Prostaglandin E_2 can also suppress lytic activity of IL-2 activated NK cells (Linnemeyer and Pollack 1993).

The other mechanism to inhibit NK cells is probably trophoblast-derived. Results in Figure 3-1 show that trophoblast cells are generally not killed by NK cells even after IL-2 activation. It is likely that trophoblast protects itself from NK cell lysis by producing soluble or membrane-associated immunoregulatory molecules. Membrane-associated molecules could include an ovine homolog of HLA-G expressed on human trophoblast that inhibits NK lysis by interacting with CD94/NKG2A/B inhibitory NK cell receptors (Rouas-Freiss et al. 1997, Carosella et al. 1999). The sheep trophoblast may also express unrelated NK inhibitory molecules. In addition, the trophoblast is likely to produce secretory molecules that can inhibit lymphocyte function. Indeed, trophoblast conditioned medium inhibits mitogen-induced lymphocyte proliferation (Low et al. 1991). One possible immunoregulatory molecule is pregnancy-associated glycoproteins (PAG). These inactive aspartic proteases, which are secreted from ovine trophoblast between Day 15 and Day 100 of pregnancy (Xie et al. 1991, Xie et al. 1997), can inhibit proliferation of bone marrow progenitor cells (Dosogne et al. 2000).

Another important change occurring around Day 75 is the accumulation of γδ T lymphocytes in the luminal epithelium of the endometrium (Lee et al. 1992, Meeusen et al. 1993, Els et al. 2001). Majewski et al. (2001) recently demonstrated that the increase

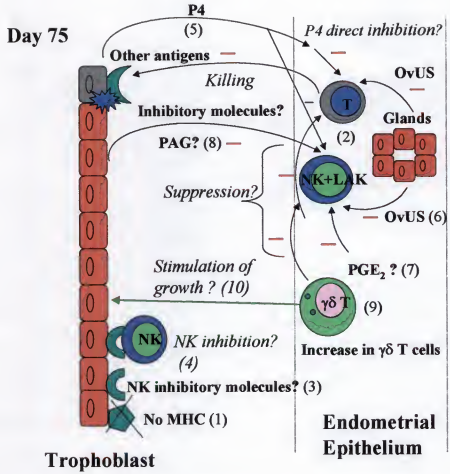

Figure 6-2. Proposed immunological status of the uterus at Day 75 of pregnancy in sheep. At this stage, there is an absence of MHC class I molecules on trophoblast (1). Nonetheless, maternal T cells (2) can sometime kill the trophoblast, suggesting expression of other transplantation antigens on trophoblast. The trophoblast is also likely to express one or more NK inhibitory molecules on its surface (3) to inhibit lytic activity of NK cells (4). At this period, the local concentrations of progesterone from the placenta are high enough to directly inhibit lymphocyte function (5). In addition, progesterone induces secretion of OvUS from endometrium and this molecule suppresses NK cell lysis and lymphocyte proliferation (6). In addition, prostaglandin E_2 (7) and PAG (8) are also likely to suppress function of NK cells and other leukocytes. The γδT cells present in luminal epithelium of the endometrium at this time in pregnancy may function to suppress activities of other lymphocytes such as NK cells (9) or to promote placental growth (10).

in γδ T cells is due to a systemic effect of pregnancy rather than to local stimulation by conceptus. That the increase in γδ T cells occurs despite the presence of OvUS is because γδ T cells appear refractory to the immunosuppressive effect of OvUS (Peltier et al. 2000c).

The exact function of endometrial γδ T cells is unclear. Meeusen et al. (1993) did not observe any signs of lysis or damage on trophoblast cells by Day 140 of pregnancy in sheep, suggesting that γδ T cells possess functions distinct from lytic activity at this period of pregnancy. Indeed, the human V2δ subset of decidual cytotoxic γδ T cells could recognize HLA-E molecules expressed on trophoblast and their cytotoxicity was inhibited via interaction of CD94/NKG2A with HLA-E (Szekeres-Bartho et al. 2000). In skin, γδ T cells play a growth-promoting role through secretion of keratinocyte growth factor (Boismenu and Havran 1994). In addition, endometrial γδ T cells express IFN-γ (Fox et al. 1998). Therefore, it is possible that γδ T cells may be involved in regulation of placental growth or endometrial vascularity (Engelhardt and King 1996). In mouse intestine, absence of γδ T cells was associated with a reduction in epithelial cell turnover and down-regulation of MHC-class II molecules (Komano et al. 1995). Endometrial γδ T cells may also be immunosuppressive. In the mouse, decidual γδ T cells inhibit lymphocytes (Suzuki et al. 1995) through secretion of TGF-β. Deletion of these γδ T cells by antibody increased abortion in the CBA/jxDBA/2 mouse mating combination (Ark et al. 1997).

A speculative model for the role of immunological events around parturition is sketched in Figure 6-3. According to this model, impending parturition is associated with a decrease in NK inhibitory molecules on the trophoblast and re-expression of MHC

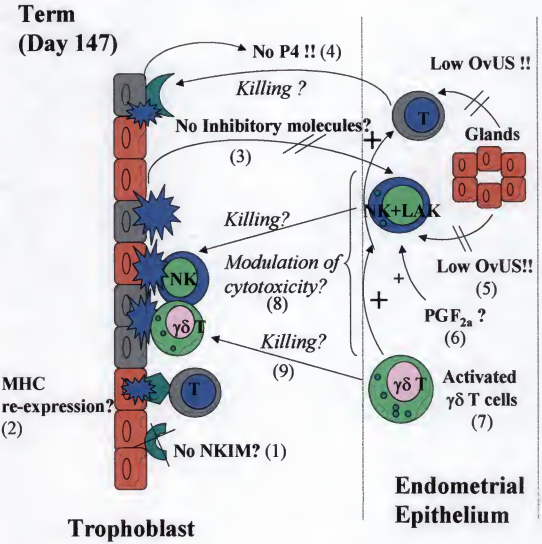

Figure 6-3. A speculative model describing the immunological status of the uterus at term (Day 147) in the sheep. According to this model, parturition is facilitated by the activation of one or more lymphocyte subpopulations that were previously inhibited earlier in pregnancy. Near parturition, it is hypothesized that NK inhibitory molecules (NKIM) (1) on trophoblast disappear. Moreover, MHC class I molecules become expressed on the trophoblast (2). A decrease in secretion of several molecules that inhibit maternal lymphocytes occurs at this time including molecules from the trophoblast (3), locally-derived progesterone (4) and OvUS (5). As a result, the inhibition to maternal T-cell and NK cell function is lifted or dampened and these cells act on the placenta to facilitate disattachment of the placenta from the endometrium. In addition, the high concentrations of $PGF_{2\alpha}$ secreted at this time may activate lymphocyte function (6). Activated γδ T cells (7) are also present in the endometrium at this time. These cells may activate NK cells (8) or directly kill trophoblast (9) to promote removal of placenta and labor.

Class I molecules. There is no evidence for this idea in sheep but in cattle, MHC class I expression on trophoblast increases during late pregnancy (Davies et al. 2000). This situation makes trophoblast susceptible to both lysis of NK cell and antigen-specific cytotoxic T lymphocytes. Indeed, susceptibility of the occasional trophoblast preparation to lysis by LAK cells (Figure 3-2) suggests that the regulatory process by which the trophoblast is protected from lysis may go wrong and lead to fetal growth retardation or death. In addition, it is suggested that concentrations of immunoregulatory molecules around parturition may not be high enough to inhibit lymphocyte function. For example, progesterone (Low and Hansen 1988, Monterroso and Hansen 1993) and PGE_2 (Linnemeyer and Pollack 1993) inhibit lymphocyte proliferation at very high concentrations (10^{-6} to10^{-5} vs 10^{-8} to 10^{-6} respectively). Around Day 120 the expression of OvUS mRNA declines (Spencer et al 2000), suggesting that concentration of OvUS around term is to low to inhibit lymphocyte function. Furthermore, Hoedemaker et al. (1992) suggested that $PGF_{2\alpha}$ can upregulate lymphocyte and neutrophil activities. Thus, concentration of $PGF_{2\alpha}$ at term may be high enough to activate NK lytic function to promote labor. The fact that endometrial γδ T cells express perforin (Fox et al. 1998, Fox and Meeusen 1999) may imply that activated endometrial γδ T cells can lyse trophoblast during parturition. Indeed, cattle CD3+ CD8- CD4- γδ T + cells lysed NK sensitive targets (Brown et al. 1994) and human uterine γδ T cells displayed NK-like activity against target cells (Jokhi et al. 1994).

In summary, the sheep possesses cells with characteristics of NK cells and the trophoblast is potentially susceptible to NK lysis. However, a combination of trophoblast and uterus-directed regulation prevents killing of trophoblast by NK cells. Thus,

regulation of NK-like cytotoxic cell is important for the maintenance of successful pregnancy. Failure of adequate regulation of NK-cell function might disrupt placental function and fetal development and lead to abortion, decreased neonatal weight, increased neonatal mortality, and reduced milk production. Future research directions could include identification of possible NK inhibitory molecules exist on trophoblast, NK inhbitory receptors on sheep NK cells, and the role of NK cells in parturition.

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BIOGRAPHICAL SKETCH

Şaban Tekin was born in 1967 in Ankara, Turkey. He graduated from Sokullu Mehmet Pasa High School and enrolled in the Department of Biology at the University of Ankara where he obtained his Bachelor of Science degree in 1989. He immediately enrolled in a graduate program in the same department and obtained his Master of Science degree in 1992. While he was obtaining his graduate degree, he taught Biology at the Ibn-i Sina High School and worked as a Biologist in the Microbiology Laboratory Ministry of Education Health Center in Ankara. In 1994, he came to the Illinois Institute of Technology where he obtained a second Master's degree in Cell Biology in 1996. Tekin continued his postgraduate study at the University of Florida, obtaining a Doctor of Philosophy in Animal Sciences within the Interdisciplinary Animal Molecular and Cell Biology Program in 2002. During his Ph.D. training, Tekin studied characterization and regulation of NK-like cytotoxic lymphocytes in peripheral blood and the uterine endometrium in sheep under the direction of Dr. Peter J. Hansen. Upon completion of his research, Şaban will continue his academic career as an assistant professor in the Department of Biology at the University of Gaziosmanpaşa in Tokat, Turkey.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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